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# The RNA Decapping Machinery is a Conserved Anti-Bunyaviral Restriction Factor

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# The RNA Decapping Machinery is a Conserved Anti-Bunyaviral Restriction Factor

## Abstract

Arthropod-borne viruses (arboviruses) are an important class of emerging pathogens that cause mortality and morbidity worldwide. As obligate intracellular parasites with limited coding capacity, viruses must hijack host factors to replicate while evading host detection. To date, no specific therapeutic interventions exist for arboviruses and most lack FDA approved vaccines. This is in part due to a lack of understanding of viral-host interactions. To identify host factors that impact infection, we performed a genome-wide RNAi screen in *Drosophila* and identified 131 genes that affected infection of the mosquito-transmitted bunyavirus Rift Valley Fever virus (RVFV). Dcp2, the catalytic component of the mRNA decapping machinery, and two decapping activators, DDX6 and LSM7, were antiviral against disparate bunyaviruses in both insect cells and adult flies. Bunyaviruses 5' cap their mRNAs by 'cap-snatching' the 5' ends of poorly defined host mRNAs. We found that RVFV cap-snatches the 5' ends of Dcp2 targeted mRNAs, including cell cycle related genes. Loss of Dcp2 allows increased viral transcription, while ectopic expression of Dcp2 impedes transcription. Furthermore, arresting cells in late S/early G2 led to increased Dcp2 mRNA targets and increased RVFV replication. Therefore, RVFV competes for the Dcp2-accessible mRNA pool, which is dynamically regulated and can present a bottleneck for viral replication.

I extended these studies to mammalian cells and I found that the two known human decapping enzymes, DCP2 and NUDT16, restrict RVFV replication. Since depletion of either gene impacted replication, this suggests that DCP2 and NUDT16 are non-redundant. In human cells, I found that RVFV predominately cap-snatches from mRNAs associated with translation, and the stability of these mRNAs is regulated by decapping; furthermore, instability of these mRNAs is triggered by RVFV infection. I hypothesize that translationally-associated genes, including ribosomal protein mRNAs, are selectively degraded during this response to limit translation to prevent viral replication. These data suggest that a particular functional class of mRNAs (translation-associated) can be coordinately regulated at the level of mRNA stability by decapping, and that this may be used as a mechanism by cells to selectively regulate gene expression.

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**THE RNA DECAPPING MACHINERY IS A CONSERVED ANTI-BUNYAVIRAL  
RESTRICTION FACTOR**

Kaycie C. Hopkins

A DISSERTATION

in

Immunology

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2013

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## **DEDICATION**

This dissertation is dedicated to my grandmother, Frances Mueller Hopkins, who taught me that all things are possible with enough curiosity, and who would've been a fabulous neuroscientist in her own right. Next time around, Ma.

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## ABSTRACT

### THE RNA DECAPPING MACHINERY IS A CONSERVED ANTI-BUNYAVIRAL RESTRICTION FACTOR

Kaycie C. Hopkins

Sara Cherry

Arthropod-borne viruses (arboviruses) are an important class of emerging pathogens that cause mortality and morbidity worldwide. As obligate intracellular parasites with limited coding capacity, viruses must hijack host factors to replicate while evading host detection. To date, no specific therapeutic interventions exist for arboviruses and most lack FDA approved vaccines. This is in part due to a lack of understanding of viral-host interactions. To identify host factors that impact infection, we performed a genome-wide RNAi screen in *Drosophila* and identified 131 genes that affected infection of the mosquito-transmitted bunyavirus Rift Valley Fever virus (RVFV). Dcp2, the catalytic component of the mRNA decapping machinery, and two decapping activators, DDX6 and LSM7, were antiviral against disparate bunyaviruses in both insect cells and adult flies. Bunyaviruses 5' cap their mRNAs by 'cap-snatching' the 5' ends of poorly defined host mRNAs. We found that RVFV cap-snatches the 5' ends of Dcp2 targeted mRNAs, including cell cycle related genes. Loss of Dcp2 allows increased viral transcription, while ectopic expression of Dcp2 impedes transcription. Furthermore, arresting cells in late S/early G2 led to increased Dcp2 mRNA targets and increased RVFV replication. Therefore, RVFV competes for the Dcp2-accessible mRNA pool, which is dynamically regulated and can present a bottleneck for viral replication.



I extended these studies to mammalian cells and I found that the two known human decapping enzymes, DCP2 and NUDT16, restrict RVFV replication. Since depletion of either gene impacted replication, this suggests that DCP2 and NUDT16 are non-redundant. In human cells, I found that RVFV predominately cap-snatches from mRNAs associated with translation, and the stability of these mRNAs is regulated by decapping; furthermore, instability of these mRNAs is triggered by RVFV infection. I hypothesize that translationally-associated genes, including ribosomal protein mRNAs, are selectively degraded during this response to limit translation to prevent viral replication. These data suggest that a particular functional class of mRNAs (translation-associated) can be coordinately regulated at the level of mRNA stability by decapping, and that this may be used as a mechanism by cells to selectively regulate gene expression.

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## **I. INTRODUCTION**

### **1. Arthropod-borne viruses**

Arthropod-borne viruses (arboviruses) are an emerging class of infectious diseases worldwide. Due to the vector-borne nature of their life cycle, arboviral infections present unique challenges to disease prevention and treatment. Climate change has increased the range of many species of mosquitoes capable of transmitting these infections, and introduction of nonnative arboviruses across the Atlantic has already occurred in at least one instance (West Nile virus) (71, 96). The absence of therapeutics or FDA approved vaccines for these diseases is driven in part by our lack of understanding of their replication strategies and host interactions, thus necessitating increased research.

Arboviruses are a major cause of human and vertebrate morbidity and mortality globally. These viruses replicate in a vertebrate host in the wild (the reservoir), often a small mammal, such as a rodent, causing limited to no pathology. Hematophagous arthropods, such as ticks or mosquitoes, then bite these infected vertebrates and become infected. The virus replicates to high titers while causing limited to no pathology in the insect. The insect becomes infected for life, infecting vertebrate hosts upon blood feeding. While this lifecycle often is limited in its pathology to both host (vertebrate) and vector (arthropod) species, incidental infections of so called “dead-end” or “off-target” hosts can occur; these infections are typically in livestock or humans, and do not result in retransmission to another biting insect, due to limited serum viral titers (139). Unlike arboviral reservoir species, incidental infections may result in severe pathology.

Three main classes of arboviruses exist: 1) Flaviviruses, such as Dengue and West Nile virus; 2) Alphaviruses, such as Sindbis virus and Chikungunya virus; and 3) Bunyaviruses, such as Rift Valley Fever virus (RVFV) and LaCrosse virus (LACV).

Interestingly, all arthropod viruses that are medically relevant to human health are enveloped RNA viruses (140).

## **2. Bunyaviruses and their replication**

Bunyaviruses are spherical, enveloped viruses, containing a tripartite negative sense RNA genome. Within the virion, the genome bound by nucleocapsid (N) protein, and the viral RNA dependent RNA polymerase (RdRp) L is also encapsidated (Figure 1.1). The viral envelope contains the glycoproteins, Gn and Gc, responsible for entry (15, 113). In addition to these 4 proteins, bunyaviruses encode up to three nonstructural proteins that are dispensable for infection in cell culture and are virulence factors (15, 54, 133).

The bunyaviral genome consists of three segments, Large (L), Medium (M) and Small (S). The L segment encodes the viral RdRp (L), while the S segment encodes the N protein and the non-structural NSs protein; some bunyaviruses (including RVFV) encode their S segment genes in an ambisense fashion. The M segment encodes one polyprotein that is processed into the glycoproteins (Gn and Gc), and depending on the bunyavirus species there may also be up to two nonstructural protein (NSm1 and NSm2) that are also processed through differential cleavage (Figure 1.1) (15).

As obligate intracellular parasites with a limited coding capacity, viruses must utilize existing cellular pathways for their replication. Arboviruses in particular replicate in disparate and evolutionarily distant hosts; as such, arboviruses provide a unique system to probe deeply conserved cellular biological processes. Bunyaviral replication has 5 main steps: 1) entry; 2) mRNA transcription; 3) protein translation; 4) genomic RNA replication; and 5) egress (Figure 1.2). Bunyaviruses bind to largely unknown receptors and enter through endocytic pathways. Some are thought to enter through clathrin-



mediated endocytosis, while for others macropinocytosis has been shown to be important. Uncoating and viral fusion with the membrane are pH dependent and require endosomal acidification (45), but the exact requirements and pathways for bunyaviral entry are poorly understood (80). Following entry, the viral genome is transcribed in the cytosol by N and L into viral mRNA through a mechanism known as “cap-snatching,” detailed below in Section 1.3. Transcriptional termination is determined by a short (5-6 nt) motif, and bunyaviral mRNAs are largely not polyadenylated (8). Some bunyaviral mRNAs have been shown to have stem-loop structures at their 3' ends that facilitate translation and are thought to be similar to the stem-loop structure required for replicating histone mRNA translation. However, this structure is not present in all segments, which makes it unclear how those mRNAs are translated (8, 12). Interestingly, translation of viral mRNAs occurs co-transcriptionally, whereby translational elongation prevents premature mRNA transcriptional termination (7). mRNAs synthesized from the S and L segments are translated on cytoplasmic ribosomes, while M segment mRNA is translated by ribosomes associated with the rough endoplasmic reticulum; M segment derived proteins mature in the golgi (55). Genome replication occurs through the transcription of an antisense cRNA intermediate, from which the genome is amplified. Genomic replication is thought to occur on Golgi derived tube-like membrane invaginations (48). Following genome encapsidation by N, assembly occurs at the golgi membrane, potentially through interactions between N and the intracellular terminus of Gn or Gc (55). Virions bud into the golgi, where they utilize the cell's secretory pathway for release via exocytosis.

### **3. Cap-snatching as a mechanism of viral mRNA transcription**

Eukaryotic mRNAs recruit ribosomes for translation and enhance their stability using two structures: a 5' cap encoded by a methylated guanosine attached in a 5' to 5' orientation via a triphosphate bond, and a 3' polyadenosine tail. All viruses utilize host ribosomes for translation and thus must recruit host ribosomes. Viruses that transcribe their mRNA in the nucleus, such as Human Immunodeficiency virus (HIV), hijack the cellular capping and polyadenylation pathways to make their mRNAs indistinguishable from endogenous messages. Cytoplasmically replicating viruses have evolved diverse mechanisms to recruit host ribosomes and to protect their 5' and 3' RNA ends from degradation. First, some cytoplasmically replicating RNA viruses, such as Sindbis virus (SINV) and Vesicular Stomatitis virus (VSV), use their RdRps to cap and polyadenylate their mRNAs (2, 74). Second, some viruses, including the arthropod-transmitted flaviviruses, lack poly-A tails, but use nonstructural proteins to create 5' caps and recruit ribosomes through use of internal ribosome entry sites (IRES), sequences that form elaborate secondary structure to facilitate ribosome assembly (124). Third, negative sense segmented RNA viruses have all evolved a mechanism of 'cap-snatching,' in which the 5' cap and a short sequence of a host mRNA is cleaved by the viral RdRp and used as a primer to initiate transcription of viral mRNAs (50).

Segmented negative sense RNA viruses include the orthomyxoviruses, the arenaviruses and the bunyaviruses. Best studied of these are the orthomyxoviruses, in particular Influenza A virus, primarily due to its drastic impact on human health. Orthomyxoviruses transcribe their mRNA in the nucleus and cap-snatch off of pre-mRNAs that are not fully spliced (59, 102). Unlike Influenza, bunyaviruses and arenaviruses transcribe their mRNAs in the cytoplasm and thus they must use a distinct pool of host mRNAs. Bunyaviruses recognize 5' caps of host mRNAs using specific binding sites on the N protein, and their polymerase encodes endonuclease activity that

cleaves 10-18 nt downstream of the 5' cap (17, 85, 100, 106, 116). This primer is then used to initiate viral mRNA transcription (Figure 1.3). Therefore all bunyaviral mRNAs have 10-18 nucleotides (nt) of nonviral origin at their 5' end (116). The pool of endogenous mRNAs that are snatched by bunyaviruses is not known; however data from Mir et al. demonstrated that ectopically expressed mRNAs incorporate into viral mRNAs, and that this incorporation is increased if a nonsense-mediated decay (NMD) signal is introduced into the mRNA (83). This suggests that bunyaviral cap-snatching may interact with the host RNA decay machinery, but it is unknown how this may occur.

#### **4. Rift Valley Fever virus**

Bunyaviruses are a large diverse group that is divided into 5 genera: rodent-transmitted hantaviruses (the only genus lacking an arthropod vector); plant-infecting tospoviruses transmitted by thrips; tick-transmitted nairoviruses; mosquito-transmitted orthobunyaviruses (e.g. La Crosse virus); and the phleboviruses, which are primarily transmitted by biting flies, but some of which (e.g. Rift Valley Fever virus) are mosquito-transmitted (135). In the orthobunyavirus genus, La Crosse virus is endemic to the United States and can cause severe pathology in the young, elderly and immune susceptible, including encephalitis and death (52, 56). Hantaviruses, which are the only genus of bunyaviruses transmitted through aerosolization of rodent excreta rather than biting arthropods, are highly fatal and cause severe pulmonary pathology in infected humans; recent outbreaks of the hantavirus Sin Nombre virus in the United States have caused fatalities (1). The emerging phlebovirus, Rift Valley Fever virus (RVFV) is considered a select agent by the USDA and Human Health and Services and has been considered to be a bioterrorist threat (140). RVFV typically causes self-limiting febrile illness in humans, but it can cause fatal hemorrhagic or encephalitic disease in 1-3% of

patients and has high mortality rates among livestock (13, 101). Unlike most other viruses in the phlebovirus genus, which are tick-transmitted, RVFV is transmitted by mosquitoes. RVFV is endemic in Africa but has recently spread to the Arabian peninsula (47); mosquito species elsewhere, including in the US, have been shown to be capable of carrying and transmitting RVFV, highlighting the importance of this pathogen to human health, both worldwide and domestically (47). Additionally, the natural vertebrate reservoir of RVFV is unknown. Infection of humans occurs either by the bite of a mosquito or through the handling of infected livestock (47). There are no commercially available vaccines or specific treatments for RVFV. This is due in part to our lack of understanding about the interplay between this virus and cellular host factors.

## **5. RNAi screening as a method to identify host-pathogen interactions**

The advent of technology allowing for the artificial use of the RNA-interference (RNAi) pathway to target specific messages has opened the door for new approaches to loss of function screening. High-throughput cell based screening technology has led to an explosion of unbiased RNAi screening to identify host factors that pathogens subvert or hijack. In addition to allowing for the identification of novel therapeutic targets, these studies have also led to a greater understanding of cellular biology, including basic cellular mechanisms of viral recognition and limiting factors for viral replication (29).

*Drosophila* provides an excellent model for unbiased RNAi screening for conserved host factors impacting viral pathogens (34, 58, 88, 107, 108, 112). In addition to their high efficiency of RNAi knockdown, their limited genomic redundancy as compared to mammals allows for a greater chance of identifying potential targets based on a single gene knockdown approach. Additionally, in tissue culture, there is no need to transfect to perform RNAi on *Drosophila* cells, decreasing the rate of knockdown

variability and cost. Using *Drosophila* as a model also allows for ease in transitioning to *in vivo* approaches for confirmation of screen hits, as comprehensive mutant and *in vivo* RNAi lines are commercially available and have short generation time (27, 28, 33). Technically, the long-term use of fruit flies as a model has also led to an exceptionally well-annotated genome, and almost complete genomic coverage can be obtained commercially for RNAi screening. Finally, *Drosophila* has been shown to share conserved immune factors and pathways with higher organisms, including humans (e.g. Toll) (72), and thus provides an excellent model for probing conserved interactions with viral pathogens.

## **6. RNA degradation**

The steady-state levels of RNAs are driven by two competing pathways: biogenesis and decay. In Eukaryotes, mRNA stability and translation are intricately linked by both cis and trans elements. At the 5' end, the RNA cap protects against 5' to 3' decay by exonucleases and promotes translation. Likewise, at the 3' end, the polyA tail recruits Poly A Binding Protein (PABP), which prevents 3' to 5' decay and promotes translation (127). Furthermore, proteins bound to these elements promote RNA circularization, which also facilitates translation initiation. Therefore, to degrade an mRNA, these protective elements must be removed. Deadenylation is thought to be the initial signal triggering most RNA turnover and is thought to be preceded by or concomitant to decreases in the rate of translation. Subsequently, RNAs can be degraded from either end. The RNA exosome is a large complex that includes two exonucleases and is the major 3' to 5' exonuclease in the cell. 5' to 3' decay first requires the removal of the 5' cap, and this step is rate limiting for decay. mRNA decapping occurs on aberrant pools of mRNAs in the nucleus, as well as on

deadenylated mRNAs in the cytoplasm. Canonical mRNA decapping itself is catalyzed by the NUDIX domain of DCP2, which cleaves the RNA cap, leaving an RNA moiety with a 5' monophosphate. This 5' monophosphate is the substrate for processive degradation by the 5' to 3' exonuclease XRN1 (Figure 1.4) (91). Recently, increasing complexity in decapping has been described in mammals with the identification of NUDT16, another NUDIX domain containing decapping enzyme with widespread tissue expression in mice and humans (118). Initial reports suggest that these decappers have both redundant and specific functions in specialized RNA decay pathways, such as RNA silencing, NMD decay and AU-rich-element (ARE) mediated decay (76). Furthermore, activators of decapping have been identified, including the human DEAD box RNA helicase DDX6/Rck. Many of these proteins are RNA binding and inhibit translation; as translation and decapping are competing steps, these activators tip the balance in favor of decay (35, 46, 122). However, the exact mechanisms of decapping activation are unknown (94, 142).

## **7. Processing bodies**

Processing (P) bodies are recently described (43) cytoplasmic aggregates of RNPs that contain most of the RNA decay machinery. These RNP complexes are endogenously present and are dynamically regulated; microscopically visible P bodies fluctuate with various cellular stresses and conditions (42). P body granules are thought to contain all of the RNA silencing, non-sense mediated decay and 5' to 3' RNA degradation machinery (64, 114, 129). Unlike closely related stress granules, ribosomes are absent from P body complexes (41). Changes in P body architecture have been described following knockdown or overexpression of various components (42). Further, visible punctae integrity is dependent not only on particular nucleating proteins, but also

on the endogenous RNAs in these complexes (42). Interestingly, while silencing and decapping components are in P bodies, the presence of microscopically visible punctae is not required for their activity (42). This suggests that while P body architecture may be informative, it is not be physiologically required for some cellular activities occurring in P bodies (42). Furthermore, some RNAs are thought to be stored in these structures, and RNAs targeted to P bodies sometimes re-enter pools of translating mRNAs (4, 99). For example, specialized cell types, including germ cells and neurons, have P body-like structures that share many protein components with P bodies (97, 141). These RNP granules are thought to be sites of mRNA storage and to allow for the control of mRNA translation in a spatially and temporally regulated manner. Increasing electron microscopy evidence also suggests that P bodies and specialized P body structures are in close proximity with ribosomes (36, 141), although whether this close association facilitates the return of P body mRNAs to translational pools or is the result of incoming mRNAs being targeted for degradation is not clear.

## **8. Aim of present studies**

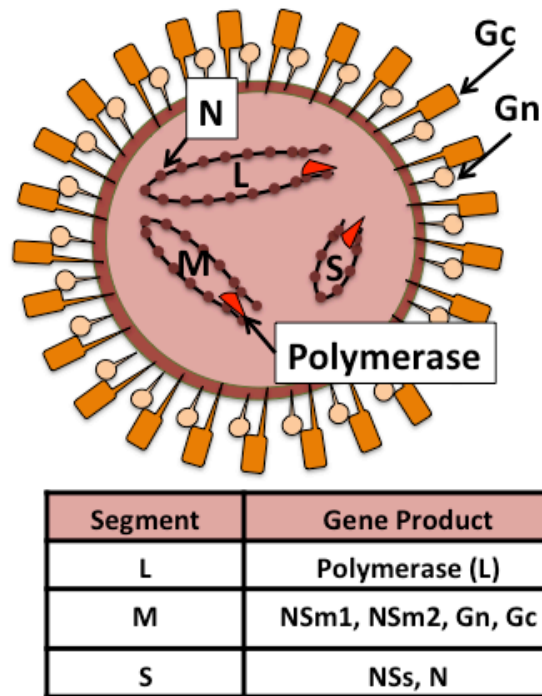
Rift Valley Fever virus has shown the potential to spread as it has emerged; originally isolated to Sub-Saharan Africa, RVFV has spread to cause outbreaks in Egypt and the Saudi Arabian Peninsula in the last few decades. As mosquitoes throughout the United States are permissive to infection, and since its impacts on human health and livestock could be catastrophic, increasing our understanding of the molecular mechanisms of RVFV replication is essential to the development of therapeutics and vaccine strategies. Therefore, the work described in Chapter III aims to characterize the host-pathogen interactions of RVFV using a cell-based genome-wide RNAi screen in *Drosophila*. The goal of these studies is to identify host-pathogen interactions, especially

those that may be conserved among multiple hosts of RVFV infection, for potential therapeutic intervention.

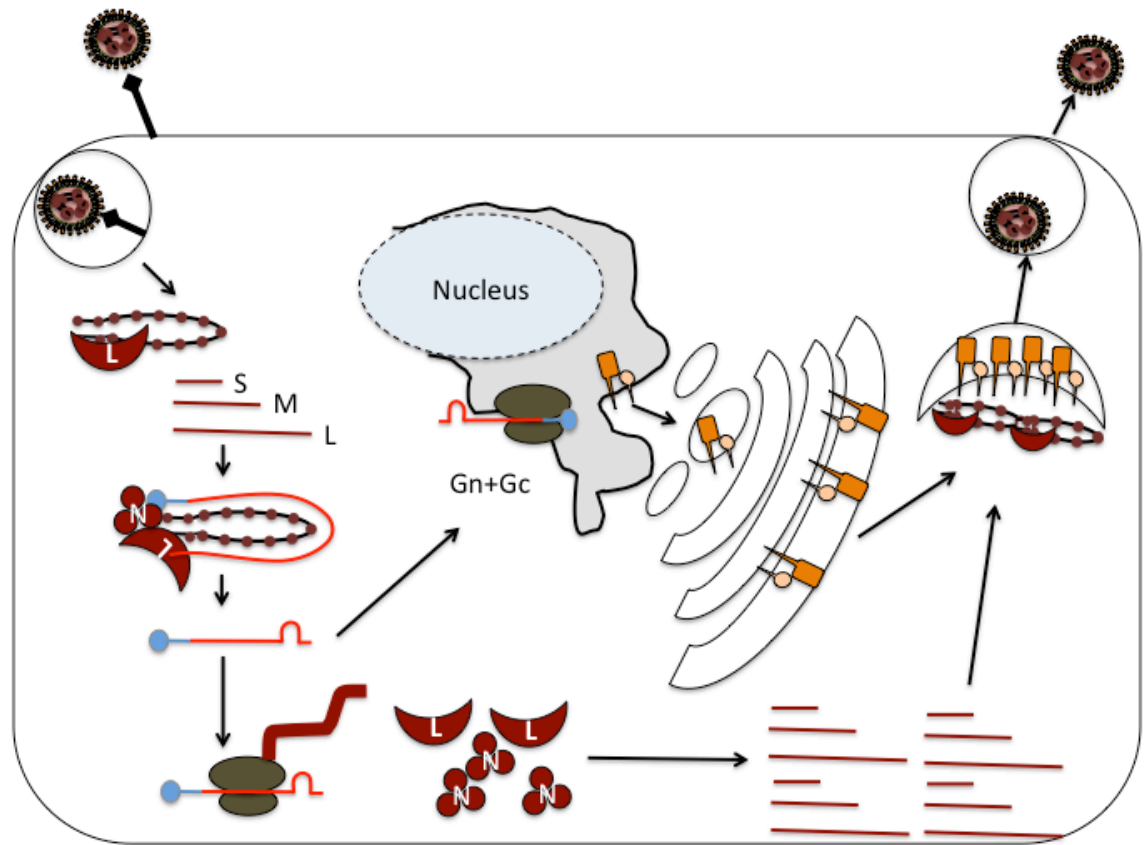
From the genome-wide RNAi screen in Chapter III, I found that three components of the P-body-resident RNA decapping pathway were antiviral in *Drosophila*. In Chapter III, I go on to characterize the mechanism by which the RNA decapping enzyme Dcp2 restricts RVFV replication and find that this restriction is bunyaviral specific and occurs at the level of cap-snatching. I demonstrate that RNA decapping and cap-snatching are competing processes for the same pool of RNA substrates, and that cap-snatching and decapping are spatially linked in P bodies. Using sequencing strategies to examine the 5' ends of viral mRNAs, I find that cap-snatching occurs primarily on cell cycle related mRNAs in insects. In Chapter III, I also show that P body morphology and the cell cycle are linked in insects, extending the findings of others in humans to show that this is a deeply conserved process.

I next extended these studies to examine whether cap-snatching is a conserved bottleneck for RVFV infection in humans. In Chapter IV, I demonstrate that either of the characterized mRNA decappers in humans, DCP2 or NUDT16, is able to restrict RVFV infection. Additionally in Chapter IV, I set out to characterize the pools of endogenous mRNAs that are snatched by RVFV in humans; I find that RNAs related to translation are predominantly snatched during infection and demonstrate both redundancy and specificity of the decapping enzymes to degrade these RNA targets. Intriguingly, in Chapter IV, I find that RVFV infection triggers loss of microscopically visible P bodies in humans late in infection, which may be due to the activation of decapping as an antiviral response to RVFV. Thus, these studies demonstrate that mRNA decapping has a deeply conserved intersection with bunyaviral replication and provide insight into potential therapeutic targets for bunyaviral treatment in multiple host organisms.

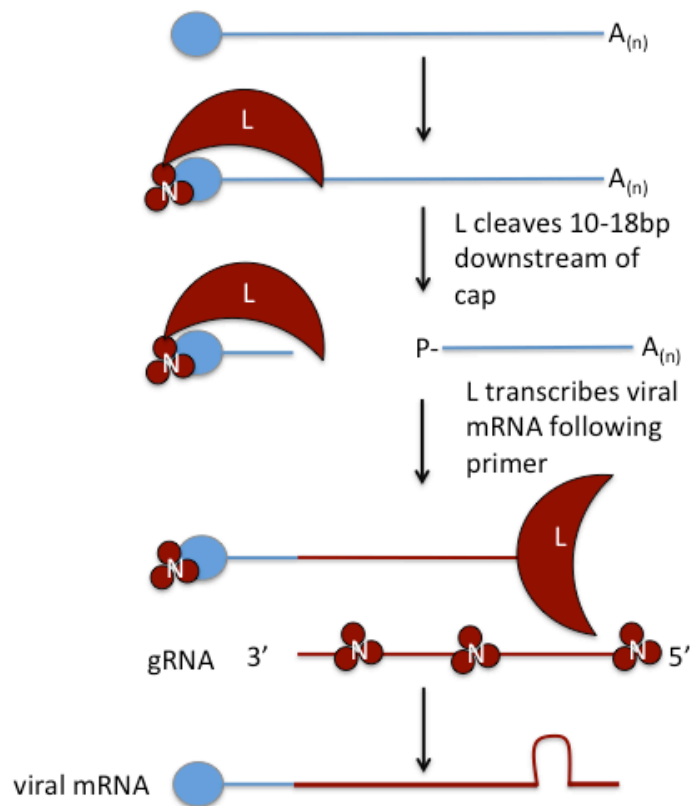




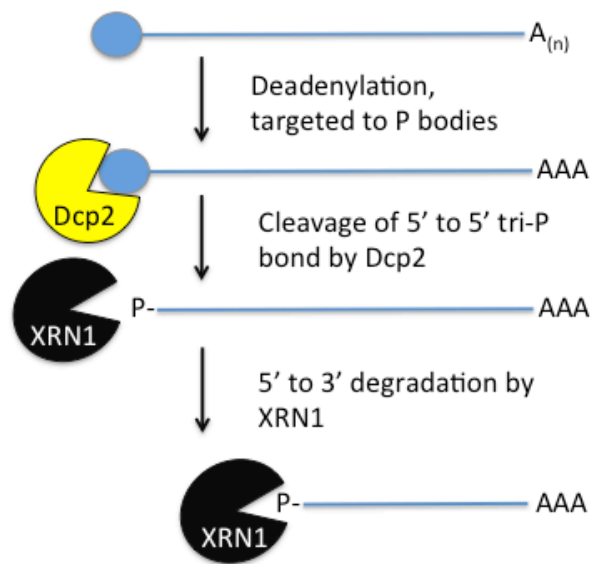
**Figure 1.1. Rift Valley Fever virus virion structure and coding strategy.** Rift Valley Fever virus virions are enveloped (dark red) and spherical and composed of four main structural proteins: the glycoproteins Gc (orange) and Gn (peach) on the envelope surface, the nucleocapsid (N) protein encapsidating the anti-sense viral genome (dark red circles), and the viral polymerase (L), which facilitates viral transcription following viral entry (bright red wedges). The viral genome is comprised of three anti-sense segments: Large (L), encoding the viral polymerase; M, which encodes two nonstructural proteins (NSm1 and NSm2) and the glycoproteins (Gn and Gc); and S, which encodes the nonstructural virulence factor NSs and the nucleocapsid protein N in an ambisense strategy.



**Figure 1.2. Bunyaviral entry, replication and assembly.** Bunyaviruses enter cells by first binding to largely unknown receptors (top left) that mediate endocytosis. Entry occurs in a pH dependent fashion. Viral mRNA transcription occurs via a cap-snatching mechanism (middle left). Viral mRNAs encoding the glycoproteins Gn and Gc are translated by ribosomes into the rough endoplasmic reticulum (middle); these proteins then mature in the golgi. Other viral proteins are translated in the cytoplasm and facilitate viral genomic replication (bottom left to right). Viral genome is encapsidated by nucleocapsid protein and buds into the golgi (middle right). Finally, fully assembled virions egress from the cell using cellular exocytosis pathways (top right).



**Figure 1.3. Bunyaviral cap-snatching.** Host mRNA caps (blue) are recognized by the viral nucleocapsid protein, which recruits the viral polymerase L. L cleaves 10-18nt downstream of the 5' cap, yielding a snatched, capped oligomer and an RNA moiety with a 5' monophosphate. L transcribes the viral mRNA from the genomic RNA downstream of this primer. Completed viral mRNAs have host derived 5'caps (blue) and lack poly-A tails; some viral mRNAs may have 3' stem-loops that aid in viral translation (red).



**Figure 1.4. 5' to 3' mRNA decay in Eukaryotes.** To initiate 5' to 3' RNA decay, cellular mRNAs are deadenylated and then targeted to the canonical decapping enzyme, Dcp2, which resides in Processing (P) bodies. Dcp2 cleaves the 5' to 5' triphosphate bond, removing the 7mG cap and exposing a 5' monophosphate. This monophosphate is the substrate for the processive 5' to 3' exonuclease XRN1.

## **II. MATERIALS AND METHODS**

### **1. Cells, Viruses, Antibodies and Reagents**

*Drosophila* DL1 cells were grown and maintained as previously described (32). Human U2OS cells were grown and maintained as previously described (88). VSV-eGFP (gift from J. Rose) was grown in BHK cells as described (105). Sindbis/GFP (gift from R. Hardy) was grown in C636 cells (21). DCV was grown and purified as described (32). MP12 strain of RVFV was grown in Vero cells as described (45). Original strain of LACV was prepared as described previously (66). Antibodies were obtained from the following sources: anti-GFP (Invitrogen), anti-tubulin (Sigma), anti-DCV (32), anti-RVFV N ID8 and anti-RVFV Gn 4D4 (gifts from R. Doms), anti-Rck (MBL), anti-Dcp1a (Abcam), anti-beta Integrin (Abcam), and Alexa-488 conjugated Rabbit anti-Flag (Invitrogen). Fluorescent secondary antibodies were obtained from Invitrogen, and HRP-conjugated antibodies were obtained from Amersham. Additional chemicals were obtained from Sigma.

### **2. RNAi**

dsRNAs were generated as described (18). For RNAi, DL1 or Aag2 cells were passaged into serum free media and plated into wells containing dsRNA. One hour later, complete media was added and cells were incubated for three days.

### **3. siRNA**

U2OS cells were seeded on 6 well plates in 2mL complete DMEM, then transfected with the indicated siRNA using HiPerfect transfection reagent in OptiMEM at

a final concentration of 20nM. Cells were incubated for three days to allow for knockdown.

#### **4. Viral Infections**

Insects: Three days post-RNAi, cells were infected with the indicated viral inoculum. VSV (MOI=0.01) and DCV (MOI=0.4) were processed at 24 hours post infection. SIN (MOI=2.5) and RVFV (DL1-MOI=0.01, Aag-2-MOI=0.06) were spinoculated at 1200 rpm for 2 hours and processed at 36 hours and 30 hours post-infection, respectively. For RNA collection, cells were infected with either RVFV (MOI=0.1) or LACV (MOI=2.5), spinoculated for 2 hours at 1200rpm, and collected at either 30 or 72 hours, respectively.

Human cells: Three days post-siRNA cells were infected with RVFV at an MOI of 1. Cells were processed at 18-22 hours post infection for all experiments unless otherwise indicated.

#### **5. Immunofluorescence**

Cells were fixed, processed, imaged by automated microscopy and subject to automated image analysis as described (107). For colocalization studies, z-stacks of 20 planes were taken at 63X, deconvolved and scored.

#### **6. RNA analysis**

Total RNA was extracted, northern blotted and quantified as previously described (30). RT-qPCR was performed as previously described (143). Primer sequences are described below in section 2.13.

## **7. Cycloheximide treatment**

*Drosophila* cells were treated with the indicated dsRNA and infected with RVFV. 28 hours post infection, cells were treated with 10µg/mL cycloheximide, and total RNA was collected every 30 minutes.

## **8. 5'RACE and cloning**

5'RACE was performed using the FirstChoice RLM-RACE kit from Ambion according to the manufacturer's instruction. RT-PCR was performed as described (143) using primers specific for the 5'RACE adaptor (forward) and RVFV N transcript (reverse) and gel purified (Qiagen) prior to ligation using TOPO TA cloning system (Invitrogen). Sequences were blasted against the *Drosophila* genome; those that matched within 40 bp of the annotated 5' end of a transcript and containing less than or equal to 1 end mismatch were considered hits.

## **9. BOWTIE analysis of human 5'RACE sequences**

Snatched sequences were mapped to the Hg19 genome with BOWTIE, using a seed length of 12 and allowing zero mismatches. The resulting regions were intersected with the coordinates of annotated human 5' UTRs, downloaded from UCSC (Hg19). The 5' UTRs were then manually inspected to verify the presence of the snatched sequence near the transcriptional start site. If multiple matches were present, distance from the annotated transcriptional start site was used to determine the more likely match.

## **10. Exonuclease Digest Assay**

10µg of total RNA was denatured at 95°C for 2', then either mock treated or digested using Terminator Exonuclease (Epicentre) as per manufacturer's instruction and evaluated by northern blot.

### **11. Adult Fly Infections**

Flies were obtained from the VDRC or Bloomington stock center. 4-7 day old flies carrying UAS-Dcp2 IR (VDRC transformant: 22272) or control (w1118) were crossed to hs-Gal4 and challenged and heat shocked every two days (RVFV) or maintained at 29°C throughout the experiment (LACV) (32). Flies were monitored daily for mortality (analyzed with a log rank test) or 15 flies per condition were processed at the indicated time point post infection for RNA analysis as described (143).

### **12. Cap radiolabeling of mRNA**

Uncapped mRNA was *in vitro* transcribed from pT GFP vector (83) using a T7 transcription system (Ambion T7 5x Megascript Kit) and then purified by precipitation. RNA was capped using Vaccinia Virus Capping System (NEB) with P32 alpha-GTP (Perkin Elmer) according to manufacturer's instruction.

### **13. Oligonucleotide Sequences for Probes and RT-PCR**

Dcp2 qPCR primers (*Drosophila*):

F 5'- CGCAAGGAGAAGCAGCAACAACTT-3',

R 5'- TGA CTGGCTGCTGTGGATTGTACT-3'

DDX6 qPCR primers (*Drosophila*):



F 5'- TCGATTTCCCACGAATGGCAGAGA-3',  
R 5'- TCCGATGCAGATCAAACCGATCCT-3'

CG8878 qPCR primers (Drosophila):

F 5'- AGGCGCTATGAAGGTAAACCCACT-3',  
R 5'- TATGCTCCTGGACAACCAAACCCT-3'

CG7580 qPCR primers (Drosophila):

F 5'- TCCAACGTCTTCATCGTTACTC-3',  
R 5'- TTATTCGTCTGTTGCGTAGTC-3'

Jupiter qPCR primers (Drosophila):

F 5'- GCTACAAGGTCGTAGCCAAC-3',  
R 5'-ACAGGCCCGACGAGTAG-3'

RP49 qPCR primers (Drosophila):

F 5'-AAGAAGCGCACCAAGCACTTCATC-3',  
R 5'-TCTGTTGTCGATACCCTTGGGCTT-3'

Dcp2 dsRNA primers (Drosophila):

F 5'-TAATACGACTCACTATAGGAGACCGGGTTCGATATCAC-3',  
R 5'- TAATACGACTCACTATAGGTCCCGCTCCCGTTCCA-3'

DDX6 dsRNA primers (Drosophila):

F 5'- TAATACGACTCACTATAGGCACTCCCGGACGAATATTAG-3',

R 5'- TAATACGACTCACTATAGGAGATTGCGACAGAGTCCTT-3'

Dcp2 dsRNA primers (Aedes):

F 5'- TAATACGACTCACTATAGGCAAAGTGTCTGGTTCCCACTT-3',

R 5'- TAATACGACTCACTATAGGGCCATTCCTTTCTGTTTGGA-3'

DDX6 dsRNA primers (Aedes):

F 5'- TAATACGACTCACTATAGGCAGTACTACGCGTTCGTCCA-3',

R 5'- TAATACGACTCACTATAGGGGCCAATTCTGTGCAGGTAG-3'

DCP2 qPCR primers (Human):

F 5'- TCCTCGAGAGGTGGAGAAA-3'

R 5'- GAGAGCCACAGCTTCAGTATAA-3'

NUDT16 qPCR primers (Human):

F 5'- GGTAGGCAGCCACTATCATTT-3'

R 5'- GCAGTCCCTGCAGCTATATT-3'

GAPDH qPCR primers (Human):

F 5'- ACCAAATCCGTTGACTCCGACCTT-3'

R 5'- TCGACAGTCAGCCGCATCTTCTTT-3'

**5'RACE primers:**

5'RACE adaptor outer primer:

F 5'-GCTGATGGCGATGAATGAACACTG-3'

5'RACE adaptor inner primer: F

5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3'

**Host mRNA-viral mRNA conjugate RT-qPCR primers:**

MP12 N R: 5'-GGGCTTGTTGCCACGAGTTAGA-3'

MP12 N F: 5'-CAAGCAGTGGACCGCAATGAGA-3'

*Drosophila* His3 5'end Forward primer (with T7 linker):

5'- TAATACGACTCACTATAGGATTGTGTTTTTC-3'

*Drosophila* CG8878 5'end Forward primer (with T7 linker):

5'- TAATACGACTCACTATAGGAAAACTGCGTG -3'

*Drosophila* Jupiter 5'end Forward primer (with T7 linker):

5'-TAATACGACTCACTATAGGTTTAGCGGCTTAC-3'

Human HIS3 5'end Forward primer (with T7 linker):

5'- TAATACGACTCACTATAGGCTATGGCACG -3'

Human H2A 5'end Forward primer (with T7 linker):

5'- TAATACGACTCACTATAGGATGTCNGGACG-3'

Human RPS3A 5'end Forward primer (with T7 linker):

5'-TAATACGACTCACTATAGGTCTGACCAGCACC-3'

Human RPL37A 5'end Forward primer (with T7 linker):

5'-TAATACGACTCACTATAGGCTTTCTGGGCTC-3'

### **III. MRNA DECAPPING RESTRICTS BUNYAVIRAL REPLICATION BY LIMITING THE POOLS OF DCP2-ACCESSIBLE TARGETS FOR CAP-SNATCHING IN INSECTS<sup>1</sup>**

#### **1. Background**

RNA stability is a key factor in the regulation of eukaryotic gene expression. Within the RNA moiety, cis elements, including the 5' 7mG cap and the 3' poly-A tail, play dual roles in protecting the mRNA from exonuclease-mediated degradation and promoting translation. RNA degradation is both actively regulated and an essential part of normal RNA turnover (127). Two strategies account for the majority of mRNA turnover: 3' to 5' mediated decay via the exosome and 5' to 3' degradation by the exonuclease Xrn1. Both strategies are dependent on loss of protective cis elements; initial deadenylation of the polyA tail signals for both exosome-dependent targeting and removal of the 5' 7mG cap by the canonical decapping enzyme Dcp2 (127). Dcp2 cleavage of the cap exposes a 5' monophosphate that is the substrate for Xrn1 (90). Furthermore, perhaps as a regulatory mechanism, the RNA degradation machinery is largely compartmentalized within the cytoplasm. The decapping machinery and the 5' to 3' exonuclease are localized to Processing (P) bodies (64, 115, 129). P bodies are granules of ribonucleoproteins (RNPs), microscopically visible, and are dynamic in their size and number. Additionally, P bodies act as storage depots; some RNAs targeted to the P body are degraded, while others may be released (99). Thus the dynamic control of mRNA stability and turnover can be regulated by P body biology. This is consistent with the fact that cellular conditions, including stress and translational inhibition, alter the

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<sup>1</sup> This chapter is reprinted from Hopkins, K., L. McLane, T. Maqbool, B. Gordesky-Gold, and S. Cherry. 2013. A genome-wide RNAi screen reveals that mRNA decapping restricts bunyaviral replication by limiting the pools of Dcp2-accessible targets for cap-snatching, *Genes & Development*, with permission from Cold Spring Harbor Laboratory Press.

visible morphology of P bodies within the cytoplasm (42). Interestingly, however, microscopically visible P body punctae are dispensable for the function of multiple mRNA decay pathways, suggesting that their structure is a marker for increased pools of accumulating mRNAs (42).

As obligate intracellular pathogens with limited coding capacity, viral RNAs must replicate to high levels and hijack the translation apparatus, while simultaneously avoiding the host's degradation machinery. Furthermore, RNA viruses must also maintain stability of different RNA species, including the genome, antigenome and mRNA. Viruses have evolved complex strategies to protect their 5' ends from exonucleases while facilitating translation. Some viruses that replicate in the nucleus hijack the endogenous capping machinery (e.g., retroviruses), while viruses that replicate in the cytoplasm cannot. To overcome this barrier, some cytoplasmic viruses encode their own capping machinery and generate mRNAs that resemble endogenous mRNAs (e.g., rhabdoviruses) (73). Other viruses protect the 5' end from degradation by covalently attaching a protein to the 5' end that prevents targeting by exonucleases (e.g., picornaviruses). However, this prevents canonical translation, and thus these viruses use internal ribosome entry sites to engage the translation machinery (110). Another group of viruses "cap-snatch," that is they steal the 5' end of host mRNAs using a virally-encoded endonuclease, generating primers that are used by the viral RNA-dependent RNA polymerase to generate viral mRNAs (51). The 5' end of the viral mRNA is therefore indistinguishable from endogenous mRNAs, and thus it is both protected from degradation and able to recruit host ribosomes. All negative sense segmented RNA viruses (orthomyxoviruses, arenaviruses and bunyaviruses) cap-snatch. Of these, Influenza A virus, an orthomyxovirus, is the best studied and snatches the 5' end of pre-mRNAs in the nucleus (59, 102). Since bunyaviruses and arenaviruses replicate in the

cytoplasm, they must use a distinct pool of mRNAs; however, while our mechanistic understanding of bunyaviral cap-snatching is increasing (25, 85, 87, 106, 130, 131), little is known about whether the host can combat this replication step or what pool of endogenous mRNAs are being targeted for this process.

Bunyaviruses are an emerging group of medically and agriculturally important viruses, many of which are insect-borne. Rift Valley Fever virus (RVFV) is a mosquito-borne emerging bunyavirus in Africa that can cause encephalitic or hemorrhagic symptoms in infected humans, leads to spontaneous miscarriage in pregnant livestock and causes high rates of mortality in young animals (13, 101). Currently there are no therapeutics or FDA approved vaccines to combat bunyaviral infection. This is in part due to a lack of understanding of the molecular interactions occurring between bunyaviruses and host cells. We set out to identify host factors that restrict RVFV infection in insects using *Drosophila* as our model insect due to the ease of genetic manipulation both *in vitro* and *in vivo*. RNAi in *Drosophila* cells is robust, and conserved immune biology with humans has been demonstrated (e.g., Toll)(72), suggesting that we can use *Drosophila* to probe both insect and human antiviral factors (26, 58, 88, 107, 108, 112). Furthermore, *Drosophila* has been used as a model to study arboviral infection, including RVFV (45, 88, 93, 107, 108, 112).

Using genome-wide RNAi screening in *Drosophila* cells, we identified 131 genes that impact RVFV (strain MP12) infection, including Dcp2, the P body resident mRNA decapping enzyme. Dcp2 restricts RVFV in *Drosophila* and mosquito cells and also in adult flies. This restriction is likely general to bunyaviruses, since the distantly related bunyavirus La Crosse virus is also restricted by Dcp2. Mechanistically, we found that the viral nucleocapsid (N) is localized to P bodies, and RVFV competes with the RNA degradation machinery for target mRNAs. Increasing the pool of mRNAs targeted for

degradation via the depletion of Dcp2 or cell cycle arrest in late S/early G2 led to increased RVFV replication, while decreasing targets via ectopic expression of Dcp2 restricted infection. Therefore, our data point to a model in which the pool of Dcp2-accessible mRNAs is dynamically regulated and presents a bottleneck for RVFV replication.

## **2. Results**

### **Genome-wide screening implicates the mRNA decapping machinery as a restriction factor for RVFV**

In order to identify host factors that restrict RVFV, we performed a high-content genome-wide RNAi screen in *Drosophila* cells. Briefly, 384-well plates were arrayed with dsRNAs targeting approximately 13,000 genes in the *Drosophila* genome (Ambion); *Drosophila* cells were seeded and knockdown was allowed to proceed for three days. Cells were then infected with the MP12 strain of RVFV (MOI= 0.25) (23), which differs by only 11 amino acids from the wild type strain ZH548, making it likely that cellular factors that impact MP12 replication will also impact wild type strains (134). 30 hours post infection (hpi), cells were stained for total nuclei and RVFV nucleocapsid (N). Automated microscopy, followed by automated image analysis was used to calculate the average percent infection per well (RVFV N positive cells/total cells) from four sites per well, and the screen was performed in duplicate. Genes with a Robust Z-score  $\geq 1.3$  or  $\leq -1.3$  in duplicate ( $p < 0.05$ ) that were non-toxic (Robust Z-score  $\geq -2$  in duplicate) were considered hits (Figure 3.1A). 179 genes were identified, amongst which 56 were part of multi-subunit complexes (e.g., ribosome, proteasome). Therefore, we chose only 1 or 2 genes per complex to verify as a representative, leaving 143 genes to validate. We generated



independent dsRNAs targeting unique regions of 143 genes from the initial set and validated 85 genes. Including the genes identified in the primary screen that were validated by another member of their complex, this comes to 131 genes. 124 genes restricted infection while 7 promoted infection (Figure 3.1B, 3.2, Table 6.1). In addition, Gene Ontology (GO) enrichment analysis indicated that these candidates were significantly enriched for genes involved in DNA replication, the cell cycle and mRNA metabolic processes (Figure 3.1C). Importantly, our gene list was also enriched for genes conserved with humans and mosquitoes; 124 were conserved with mosquitoes and humans, 4 were conserved in mosquitoes, while only 3 did not have identified orthologs in these groups. This suggests that the factors and pathways identified may have conserved interactions with RVFV replication across multiple relevant hosts.

Additionally, we validated three genes that reside in P bodies (115): the canonical mRNA decapping enzyme Dcp2, LSM7 (part of the heptameric LSM1-7 complex that participates in decapping activation) (95) and the *Drosophila* homolog of human DDX6/Rck/p54 (Me31B), which has been characterized as an activator of decapping in yeast (35, 46), although its mechanism is unclear (94, 122, 142) (Figure 3.1B, 3.2, 3.3A-C, 3.4, Table 6.1). P body components participate in multiple mRNA degradation pathways, including silencing and decapping (40), and P body morphology can be altered by the loss of specific P body components. Loss of Dcp2 leads to increased P body size, while the loss of DDX6 leads to the dispersal of P bodies in *Drosophila* (data not shown, and (42)). Furthermore, visible P bodies are not necessarily required for functional activity of P body resident proteins, including RNA silencing, nonsense mediated decay and mRNA decay (42). Therefore, we set out to elucidate whether P bodies themselves or particular functions within P bodies, such as decapping, were specifically involved in RVFV restriction. We screened 7 other canonical P body

resident proteins, including HPat (Patr-1), GW182 (gw), Dcp1 (a binding partner for Dcp2), EDC4 (Ge-1), staufer (stau), LSM14A (lsm14a, not part of the LSM1-7 complex) and EDC3, and found that none of them impacted RVFV replication greater than 2-fold, although loss of LSM14A or EDC3 led to modest, but significantly increased levels of RVFV infection (Figure 3.3C). Interestingly, LSM14A and EDC3 are mRNA decapping activators in yeast (95) and are required for DDX6 recruitment to P bodies in *Drosophila* (125), suggesting their effects may be through this mechanism. Our data suggest that while RVFV replication is restricted by mRNA decapping (Dcp2 and four decapping activators), P body integrity is not essential for this restriction or for RVFV replication.

### **Dcp2 restricts the bunyavirus RVFV but not other families of RNA viruses**

In order to determine the specificity of this restriction, we tested whether Dcp2 impacts the replication of RNA viruses from three disparate families: *Drosophila* C virus (DCV), Sindbis virus (SINV) and Vesicular stomatitis virus (VSV). DCV is a positive sense RNA picorna-like virus and natural pathogen of *Drosophila* (32, 67). DCV does not use a 5' cap for translation but rather couples a protein to the 5' end for protection and uses internal ribosome entry sites for translation (110). SINV is a human arbovirus that is a positive sense RNA alphavirus, and VSV is an arbovirus that is a negative sense single stranded RNA rhabdovirus; both of these viruses encode their own 5' capping machinery (2, 73). We found that Dcp2 specifically restricts RVFV, since depletion of Dcp2 has no impact on the level of infection by DCV, SINV or VSV (Figure 3.3B). Since the only known role for Dcp2 is in decapping, and it selectively restricted RVFV, we hypothesized that decapping *per se* specifically limits RVFV replication through this biological function.

## **RVFV N associates with P bodies**

Since we identified P body resident proteins as antiviral against RVFV, we tested whether the viral replication machinery and P bodies interact during RVFV infection. Previous studies of the distantly related bunyavirus Sin Nombre virus found that the nucleocapsid protein (N), necessary for cap-snatching, forms visible punctae in mammalian cells that colocalize with the P body resident protein DCP1a (83). We generated cells expressing Dcp1-GFP, which labels P bodies (42), and infected them with RVFV for 30 hours. Analysis of these cells demonstrated that N punctae partially overlapped with P body punctae (Figure 3.5A). More than half of the infected cells presented with at least one colocalization (Figure 3.5B). Furthermore, the majority of these events presented with partial overlap rather than complete colocalization (only 3 colocalizations were found to be coincident), resembling previous reports showing that P bodies may contain distinct compartments (111, 141). Additionally, colocalization studies examining P bodies and the Ty3 retrotransposon in yeast have shown similar patterns of partial overlap (9). Since Dcp2, but not Dcp1, is antiviral (Figure 3.3C), we next examined whether Dcp2 colocalized with RVFV N. We generated cells expressing myc-Dcp2 (65) and infected them with RVFV for 30 hours. We found that coincident colocalization occurred in ~90% of infected cells (Figure 3.3D, E), while only a small subset of cells showed instances of partial overlap between RVFV N and Dcp2 punctae (~2%). Interestingly, when we co-expressed Dcp1-GFP and myc-Dcp2, we saw a spectrum of overlap where the large majority of punctae had substantial overlap of Dcp1 and Dcp2, while others showed partial overlap or no overlap (Figure 3.5C). This suggests complexity to these compartments. Taken together, our data suggest that RVFV, and perhaps bunyaviruses in general, interacts with P bodies or P body resident proteins.

## **Dcp2 restricts bunyavirus infection in adult flies**

We set out to determine the role of Dcp2 in antiviral defense against RVFV at the organismal level in adult flies (45). Arboviral infection of the insect host is controlled by the innate immune system. If compromised, an otherwise non-lethal infection can become pathogenic and potentially fatal (108, 132, 136). Since Dcp2 is an essential gene (79, 103), we used *in vivo* RNAi technology to deplete Dcp2 post-developmentally in adult flies to determine the impact of the decapping machinery on RVFV replication. Briefly, transgenic flies bearing a UAS element driving the expression of an inverted repeat (IR) against Dcp2 were crossed to flies expressing the Gal4 protein under the control of a heat shock inducible promoter. Adult flies were subjected to heat shock, driving expression of the snap-back transgene and mRNA depletion, which had no impact on survival (Figure 3.5A, B). As expected, control flies infected with RVFV presented with little mortality; however, Dcp2-depleted flies succumbed to RVFV infection (Figure 3.6A). This increase in mortality was accompanied by increased levels of viral replication as measured by northern blot at 6 days post infection (Figure 3.6B, C). We also examined viral RNA levels at 20 days post infection, when flies are dying from infection, and found that while we see less of an increase in Dcp2 depleted flies compared to day 6, increased replication continues late into infection (Figure 3.7C, D). We hypothesize that the differences we see are greater at day 6 due to highly infected flies in the population succumbing to infection by day 20 post infection. Additionally, we tested whether decapping restricted another distantly related bunyavirus, La Crosse virus (LACV), an orthobunyavirus transmitted by mosquitoes to humans (53, 57). Control flies challenged with LACV presented with little mortality, while Dcp2-depleted flies succumbed to LACV infection (Figure 3.6D). Additionally, we found increased viral RNA

levels in flies infected with LACV as measured by quantitative RT-PCR 6 days post infection (Figure 3.7E). Thus, Dcp2 restricts bunyaviruses both in cell culture and *in vivo* in adult flies.

### **Decapping restricts RVFV in *Aedes aegypti* mosquito cells**

RVFV is unusual among arboviruses in that it has been isolated in nature from a large number of mosquito species, and numerous mosquitoes can experimentally transmit this virus, including *Aedes aegypti*, the major vector for Dengue virus (128). Since there are no cell lines available for many of the more common vector mosquitoes that transmit RVFV, and their genomes have not been sequenced, we took advantage of the fact that *Aedes aegypti* is a sequenced vector mosquito. Aag-2 cells, derived from these mosquitoes, are permissive to RVFV and amenable to robust RNAi (Figure 3.6E) (22, 89). There are two annotated Dcp2 orthologs in the *Aedes aegypti* genome (AAEL015607 and AAEL000783) with 99% amino acid sequence identity, allowing us to design a single dsRNA targeting a conserved region of both Dcp2 genes to deplete both simultaneously. Aag-2 cells were subject to RNAi using the same method as with *Drosophila* cells. Three days post RNAi cells were challenged with RVFV (MOI of 0.06) for 24 hours, fixed and stained for total nuclei (blue) and RVFV N protein (green). Automated microscopy and automated image analysis revealed a significant increase in the percentage of RVFV infected Dcp2-depleted mosquito cells (Figure 3.6E, F), suggesting that decapping is a conserved mechanism of bunyaviral restriction among insects.

### **Dcp2 does not restrict RVFV by directly decapping viral mRNAs**

One major difference between the replication strategies of segmented negative sense viruses (RVFV, LACV) and non-segmented negative sense viruses (VSV) is the mechanism by which they cap their viral mRNAs. While VSV encodes its own 5' capping machinery (VSV L) (73), RVFV does not. Rather, all segmented negative sense RNA viruses (bunyaviruses, arenaviruses and orthomyxoviruses) "cap-snatch" the 5' ends of host mRNAs, simultaneously defending the 5' end of their mRNAs from exonucleases and facilitating translation. Bunyavirus encoded nucleocapsid (N) protein specifically binds the 5' caps of host mRNAs (85). The viral RNA-dependent RNA polymerase (L), which has endonucleolytic activity (100, 106), then cleaves host mRNAs 10-18 nucleotides downstream of the 5' cap and uses this primer as a template for viral mRNA transcription (17, 100, 116). Thus, all viral mRNAs from this family (RVFV encodes 4 mRNAs (Figure 3.8)) begin with a short sequence of nucleotides of non-viral origin.

We reasoned that if Dcp2 restricts RVFV replication at the step of cap-snatching, there are 3 likely mechanisms: 1) Dcp2 may be restricting the pool of available host mRNAs through normal metabolic turnover (indirect mechanism); 2) following cleavage by the viral L protein, short host-derived primers may be decapped prior to viral RNA transcriptional elongation; 3) Dcp2 may be directly decapping host mRNA-viral mRNA conjugates following or concomitant to viral transcription (direct mechanism). The second mechanism is highly unlikely; following cap-binding and cleavage the 5' cap is bound by viral proteins and thus likely occluded from Dcp2 recognition (84), and in yeast, Dcp2 has been shown to be largely inactive on mRNAs <30bp (120).

In order to clarify which of the other mechanisms restricts RVFV, we took two approaches: we first assayed the cap status of viral mRNAs in the presence or absence of Dcp2, and second, we examined the decay rate of viral mRNA species in the presence or absence of Dcp2. First, we developed an assay to distinguish capped

mRNAs from those that have been decapped by Dcp2, which leaves a 5' monophosphate on mRNAs following cap cleavage (90). Terminator Exonuclease degrades 5'-monophosphate bearing RNAs, including those which are the product of Dcp2-mediated decapping. Cellular 28S rRNA, which natively has a 5' monophosphate, was completely digested by this enzyme (Figure 3.9A, B), while the capped mRNA dRPS6 remains largely intact (95% protected, Figure 3.9A, C, D). We also analyzed the level of background digestion that occurs in this assay by radiolabeling the cap of GFP mRNA transcribed *in vitro* and then subjecting these capped mRNAs to digestion in the presence or absence of cellular mRNA, as this is the context of our experimental samples. We found that without cellular RNA, ~18 percent of the GFP signal was lost in digested samples compared to mock controls. Interestingly, this level of digestion was reduced to ~12 percent with the addition of cellular RNA, presumably due to the abundance of rRNA competing for digestion (Figure 3.10A).

If Dcp2 were directly decapping viral mRNAs post cap-snatching, loss of Dcp2 would increase the proportion of undigested viral mRNA (capped) present in the viral RNA pool. Conversely, if Dcp2 were decapping the mRNA substrates available for RVFV to snatch from, there would be no change in the relative proportion of mRNA that was digested, even though there would be an increase in overall viral mRNA levels.

Since the N and NSs mRNAs are significantly different in size from their genomic segment, this allows us to distinguish them from the S genomic RNA. In contrast, the other viral mRNAs are not sufficiently different in size from the genomic RNA to distinguish by northern blot (Figure 3.8). Thus, we chose to examine the N transcript since it is essential; NSs is dispensable for replication in cell culture (14). We found that ~75% of the viral N mRNA was protected from digestion in control cells, presumably by a 5' cap (Figure 3.9F); accounting for background levels of digestion, this suggests that

anywhere from 13-25% of the viral mRNA is uncapped natively. Moreover, we observed that Dcp2 knockdown leads to an overall increase in viral N mRNA levels (Figure 3.9A, E); however, Dcp2 knockdown did not significantly alter the proportion of protected N mRNA (Figure 3.9A, F). This suggests that Dcp2 does not directly decap viral mRNAs. Furthermore, we found that Dcp2 does not seem to regulate the steady-state levels of the housekeeping mRNA dRPS6, as there is no change in dRPS6 mRNA in the presence or absence of Dcp2, with or without digestion (Figure 3.9A, C, D). These findings suggest that Dcp2 restricts the RNA substrates available for RVFV-mediated cap-snatching and does not globally affect the cap status of mRNAs within the cell. This is consistent with the finding that Dcp2 does not globally regulate mRNA turnover, but impacts the stability of only small subsets of mRNAs from yeast to humans (75, 77, 146). Furthermore, depletion of the P body resident 5' to 3' exonuclease Xrn1, does not impact viral infection levels, N transcript levels, or digestion assay results, suggesting that viral mRNAs themselves are not under considerable pressure from the 5' to 3' degradation pathway (Figure 3.10 B, C, D).

Since there was a degree of background digestion occurring with the exonuclease assay, we set out to directly determine whether Dcp2 impacted viral mRNA stability. Unlike cellular mRNAs, whose decay can be measured by treatment with Actinomycin D and subsequent monitoring for loss of mRNA, viral RNA dependent RNA polymerases are refractory to Actinomycin D preventing the use of this approach. However, a unique feature of bunyaviruses is that transcription of mRNAs is coupled to translation (7); therefore, treatment with cycloheximide (CHX), which inhibits translational elongation, prevents viral mRNA transcription, allowing us to examine the rate of viral mRNA decay.



For these studies, we infected dsRNA treated *Drosophila* cells with RVFV for 28 hours, and then treated cells with CHX. We collected total RNA every 30 minutes and examined viral mRNA levels via northern blot (Figure 3.9G). RVFV N mRNA has a half-life of ~30 minutes (Figure 3.9H). Furthermore, RVFV N mRNA decayed at the same rate in Dcp2-depleted and control cells (luciferase) (Figure 3.9H), suggesting that Dcp2 is not affecting viral mRNA stability. Furthermore, we observed no changes in viral genome levels over time with CHX treatment in the presence or absence of Dcp2, indicating that short-term disruption of translation does not globally impact the stability of other RNA species (Figure 3.9I). Altogether, these data suggest that Dcp2 is not directly decapping viral mRNAs, but rather decaps specific pools of mRNA that are the preferential targets of RVFV cap-snatching.

### **RVFV selectively snatches cell cycle related mRNAs**

As our results suggest that Dcp2 and RVFV compete for the same pool of mRNA targets, we set out to determine which mRNAs were being snatched. This may also reveal the particular mRNAs that are regulated by Dcp2-dependent decapping. Thus, we performed 5' RNA ligase mediated (RLM)-RACE and sequenced the 5' ends of viral N mRNA transcripts from RVFV infected *Drosophila* cells. Of the 40 sequenced reads, we were able to align 33 to the 5' end of endogenous RNAs (Figure 3.11A, 3.8). Of these 33, we found 4 instances in which we found the same gene being snatched in independent experiments, leaving 29 independent genes. There were no obvious consensus sequence motifs within this gene set, suggesting that if there are structural or sequence specific motifs targeted by Dcp2, they are not contained within the first 15 nucleotides. We found that while 9 of the 29 genes had no annotated GO terms, half of

the remaining 20 genes (10) had terms associated with the cell cycle and mitosis (Figure 3.11A, 3.12).

To determine whether the stability of these target mRNAs is impacted by Dcp2, we performed RNAi in *Drosophila* cells and assayed host mRNA levels by quantitative RT-PCR. We found that indeed, mRNA levels for three genes tested (CG8878, CG7580 and Jupiter) were increased upon Dcp2 depletion (Figure 3.11B). To determine if these genes were used as a target for RVFV snatching, we used a forward primer containing the first 11bp of either CG8878 or Jupiter, in addition to another gene identified, Histone 3 (His3), a replication-dependent histone, and a reverse primer in the N transcript. Using this assay, we found that the viral Host-N fusion mRNAs were increased upon Dcp2 knockdown as compared to control (Figure 3.11D). These data suggest that Dcp2 affects RVFV replication primarily by restricting the substrate mRNAs available for cap-snatching, and that cell cycle mRNAs are targeted both by decapping and cap-snatching.

### **P body morphology is regulated by the cell cycle**

Interestingly, previous studies in human HeLa cells suggested the rapid turnover of replicating histone mRNAs (such as His3) at the end of S phase is dependent on Dcp2 (92, 121). This observation, along with our identification of a large number of cell cycle related mRNAs as targets of RVFV-dependent cap-snatching, suggests that cell cycle related mRNAs are degraded by Dcp2 in *Drosophila*. Emerging data also suggest that P bodies are regulated by a number of different biological inputs, including the cell cycle. P body size and number increase as mammalian cells exit S phase and enter G2 (145). This may be due to the influx of mRNAs encoding DNA replication machinery and histones that need to be degraded, and this accumulation may result in increased

granule assembly leading to increased P body size. We examined whether P body size or number was influenced by the cell cycle in *Drosophila*. We used RNAi against cyclins to arrest the cells in G1 (CycD knockdown) or late S/G2 (CycA knockdown) (11). As expected, we observed an increase in nuclei size upon S/G2 arrest (Figure 3.11E). This was concomitant with a significant increase in P body area and number (Figure 3.11F, G), suggesting that cell cycle dependent regulation of P bodies is deeply conserved, and that P body size during the cell cycle may serve as a marker for the load of mRNAs destined for degradation.

### **Cell cycle arrest in late S/early G2 enhances RVFV replication**

Since P body size is regulated by the cell cycle with the apex at late S/early G2, which is likely due to increased targeting of RNAs for degradation, and mRNA target levels are seemingly a bottleneck for RVFV replication, we hypothesized that RVFV may replicate most efficiently when P bodies are at their largest and P body mRNAs are in high abundance. Indeed, analysis of our validated RNAi screen gene set revealed 39 genes that had the GO term “cell cycle” and were antiviral (Figure 3.1C, 3.2). Furthermore, the entire DNA replication factor A complex (RPA2, RpA-70 and CG15220), whose depletion results in S phase arrest, was also antiviral in our screen (Figure 3.2, Table 6.1). Moreover, we found that 28 genes impacted the cell cycle arresting at S/G2, as measured by increased nuclear area upon depletion (Figure 3.2, Table 6.1), of which 26 genes were antiviral and 15 genes did not have an annotated GO term associated or literature reference to the cell cycle, suggesting that they may have a previously unknown role in the cell cycle. To validate this, we performed RNAi against a panel of genes that arrest the cell cycle at specific stages. Treatment of cells with dsRNA to arrest in S/G2 (CycA, cdc2, RnRs) led to increased levels of infection,

while arrest in G1 (CycD, cdc2c, CycE) had no impact (Figure 3.13A, B), consistent with the fact that in log phase, >80 percent of *Drosophila* cells are in G1 (18). Furthermore, we observed increases in RVFV mRNA upon loss of CycA, but not CycD, as measured by northern blot (Figure 3.13C, 3.14A), along with increased His3-N mRNA accumulation (Figure 3.13D). To determine whether this was specific for RVFV infection, we challenged arrested cells with VSV. We found that VSV replication was unaffected by S/G2 arrest, while G1 arrest by cdc2 modestly promoted infection (Figure 3.13B). Altogether, this data suggests that cell cycle arrest in late S/early G2 is advantageous for RVFV replication, and this enhancement is specific to bunyaviruses.

### **Diverse bunyaviruses are restricted by similar mechanisms in cells**

Our finding that Dcp2 restricts LACV in adult flies prompted us to test this in cell culture. Depletion of Dcp2 in *Drosophila* cells led to increased levels of LACV replication as measured by viral RNA levels; similar results were seen with DDX6 depletion (Figure 3.13E, 3.14B). Next, we tested if LACV, like RVFV, also replicated more efficiently in late S/early G2, a time when Dcp2-targeted mRNAs should be abundant. Indeed, we found that S/G2 arrest led to increased LACV RNA replication (Figure 3.13E, 3.14B). These data suggest that cap-snatching is a bottleneck in the bunyaviral life cycle and that modulation of target mRNA levels impacts the replication of diverse bunyaviruses.

### **Dcp2 is limiting: ectopic expression restricts RVFV replication**

Since loss of Dcp2 leads to increased replication, we hypothesized that enforced expression of Dcp2 may restrict RVFV replication by decapping, and thereby limiting, the pool of available mRNAs. First, we confirmed that RNAi against Dcp2 substantially depleted myc-Dcp2 in our Dcp2 expressing cells by immunoblot (Figure 3.15A). Next,

wild type or Dcp2-expressing cells were infected with RVFV, and immunoblot analysis of RVFV glycoprotein (RVFV Gn) revealed that Dcp2 knockdown increases RVFV infection (Figure 3.15A), consistent with our findings measured by microscopy (Figure 3.3A) or northern blot (Figure 3.9E). Additionally, we found that ectopic Dcp2 expression significantly restricted RVFV infection as measured by both immunoblot (Figure 3.15A) and by microscopy (Figure 3.15B). Furthermore, Dcp2 knockdown restored infection in ectopically Dcp2-expressing cells to wild-type levels (compare to luciferase treated infected cells (Figure 3.15A, B). These treatments had no effect on VSV infection (Figure 3.15C), demonstrating specificity. Therefore, Dcp2 levels define a set point for RVFV infection.

### **3. Discussion**

Our genome-wide RNAi screen identified and validated a large number of genes that restrict RVFV replication. Amongst this gene set was the canonical mRNA decapping enzyme Dcp2 and two decapping activators. The interactions between mRNA decay and viral infection are an area of burgeoning study (38, 49, 86); however, there is little known about the intersection of the RNA degradation machinery and bunyaviral infection, and so we explored this biology. Future studies will reveal the mechanistic roles the other validated factors play in viral infection. We focused on Dcp2, which specifically restricts the replication of two diverse bunyaviruses (RVFV and LACV) but not other RNA viruses, in insects both *in vitro* and *in vivo*. Bunyaviruses, unlike the other RNA viruses tested, use cap-snatching to generate the 5' end of viral mRNAs. This is in part mediated by the bunyaviral N protein, which binds to 5' capped mRNAs (85). This led us to postulate that RVFV cap-snatching competes with decapping, and suggests a

model in which the availability of mRNA substrates is rate limiting for RVFV infection. Thus, modulation of these targets can create or eliminate a bottleneck in viral replication.

Indeed, we find that Dcp2 does not directly decap viral transcripts after snatching and transcription have occurred, as knockdown does not impact the cap status of viral mRNAs (Figure 3.9F) or their stability (Figure 3.9G, H), and furthermore, that Xrn1 knockdown does not increase viral mRNA stability (Figure 3.10B, C, D). Rather, mRNA decapping during normal RNA turnover keeps the pool of available mRNA targets from which bunyaviruses can snatch at a low level. Thus, loss of the decapping enzyme Dcp2 leads to increased bunyaviral replication, and ectopic expression of Dcp2 restricts infection. Furthermore, we found that many mRNA targets that are snatched and incorporated into viral transcripts are cell cycle related. Indeed, mRNA levels in P bodies are cyclically altered in phase with the cell cycle. As cells transit into late S/early G2, P bodies enlarge to accommodate mRNA degradation of mRNAs required for DNA replication, and these cells support higher levels of bunyaviral replication. Our genome-wide RNAi screen identified a large number of cell cycle genes, including all three subunits of the DNA replication factor A complex, that arrest the cell cycle at this time as antiviral. Interestingly, however, we find that P body dispersion through knockdown of components known to be required for P body integrity does not affect RVFV replication; this is consistent with data showing that microscopically visible P bodies are not required *per se* for P body associated functions, such as miRNA silencing and NMD decay. Indeed, our data strengthen previous findings that P body morphology may be a marker for the accumulation of mRNAs destined for degradation within the cell.

We found that by modulating the pool of host mRNAs targeted for decapping, either through changes in the expression level of Dcp2, or by arresting cells in late S/early G2 phase, bunyavirus replication is affected in insects. Interestingly, mRNAs

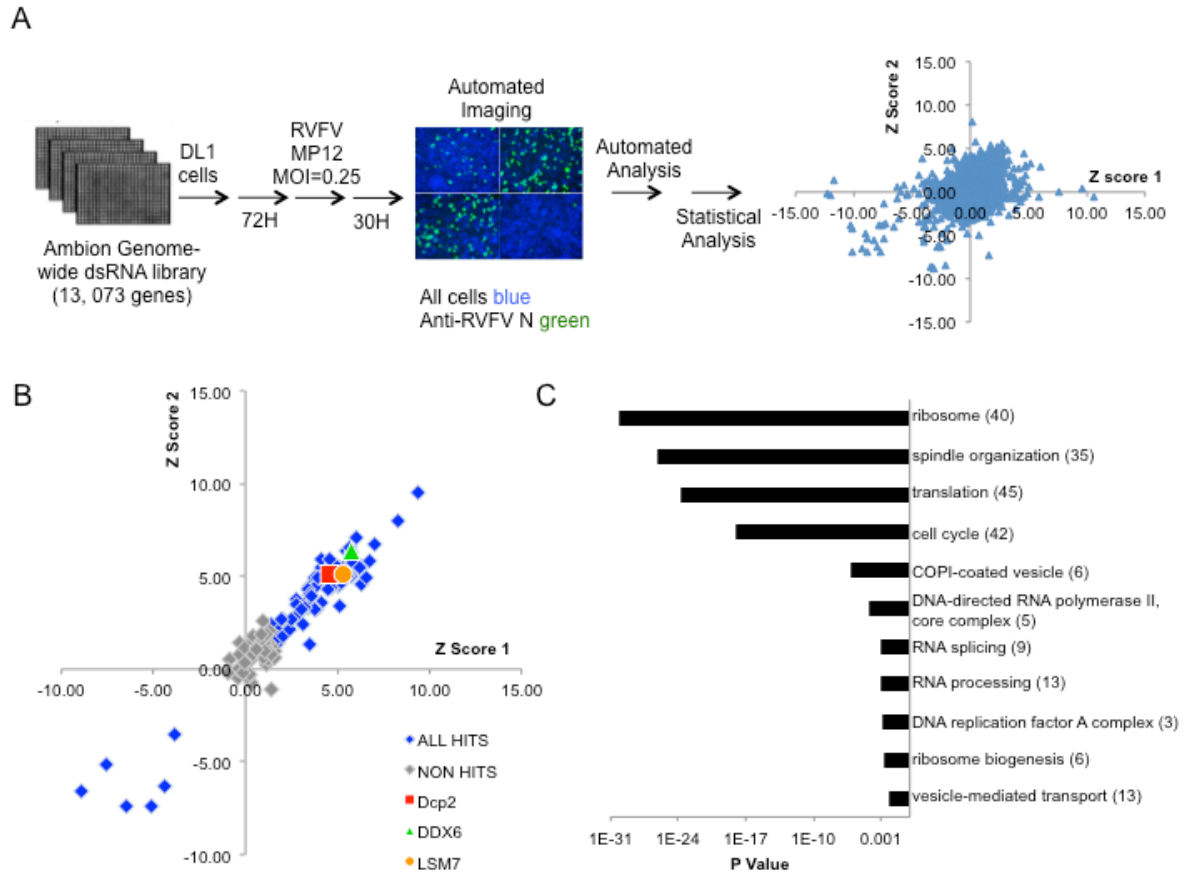
carrying nonsense mediated decay signals were incorporated at an increased rate into Sin Nombre transcripts in human cells, and Sin Nombre N localizes with P bodies (83). This suggests potential conservation of decapping as antiviral against bunyaviruses in mammals. In further support of this, Dcp2 is inducible by Type I interferons (75, 109). While *Drosophila* only encode one characterized decapping enzyme, a second decapping enzyme (NUDT16) has been recently characterized in both mice and humans (117), and an additional six nudix domain containing proteins in mice and one in yeast have been shown to have various degrees of decapping activity (119). *Drosophila* encodes no NUDT16 ortholog, however it does encode for 14 other nudix domain containing proteins, none of which were identified as antiviral in our genome-wide screen. Recent studies have identified a novel decapper in bacteria, which do not cap their RNA, suggesting that decapping activity is ancient and preceded mRNA capping in evolution (119). This may further support a role for decapping in antiviral defense. Interestingly, comparisons have revealed that DCP2 and NUDT16 have both redundant and specific targets (76), suggesting that in eukaryotes, decapping may be far more complex than first thought.

One interesting question raised by these observations is why bunyaviruses transcribe their viral mRNA in an area rife with mRNA degradation machinery. This is particularly perplexing when considering the fact that viral RNA transcription is dependent on translation, and P bodies are ribosome-free. We have four hypotheses that might explain why bunyaviruses use mRNAs destined for degradation as the target for cap-snatching. First, these targets are largely present in a spatially concentrated area and destined for degradation. Thus, snatching caps from these mRNAs should not negatively impact cell viability. Second, cytoplasmically replicating RNA viruses must compartmentalize their replication steps in order to enhance efficiency; segregating RNA

transcription to these areas (P bodies) may prevent competition between cap-snatching, genome replication and viral RNA packaging into virions. Third, microscopy studies indicate that P bodies, and perhaps the RNA degradation machinery in general, are surrounded by ribosomes (36, 141). Furthermore, recent studies in *Drosophila* have shown that during oogenesis and early egg activation, translation of mRNAs necessary for proper axis formation depends upon RNA localization within sub-compartments of P body like RNPs (141). RNAs located deep within the P body core are associated with decapping activators and are translationally repressed, while those located towards the edge of the P body are able to interact with ribosomes associated with the periphery of the P body and can initiate translation. This pool of readily available ribosomes may be optimal for initiation of translation of viral mRNAs, since bunyaviral mRNA transcriptional elongation requires translation to be occurring concomitantly (7). Thus, it is possible that these ribosomes serve this function. Finally, we speculate that the 5' end of the host mRNA molecule, once cleaved, may represent an abnormal or 'foreign' RNA structure to the host cell (5' monophosphate on an RNA with a 3' polyA tail). While cellular RNA molecules exist with 5' monophosphates, such as rRNA, these RNAs are heavily associated with proteins, which may occlude recognition of their 5' end structure, and are not polyadenylated. Snatching in these localized environments may assure that mRNAs that are targeted for snatching can be rapidly degraded by the P body resident processive exonuclease, Xrn1. Indeed, there may be parallels between bunyaviral snatching of Dcp2 targeted pools in the cytoplasm and nuclear pre-mRNAs by orthomyxoviruses. Many pre-mRNAs are aberrantly synthesized, and therefore the nucleus has surveillance machinery, which includes an Xrn1 homolog Rat1 that targets uncapped mRNAs for degradation. Therefore, in both cases the uncapped host mRNA is under close surveillance and thus has a very short half-life.

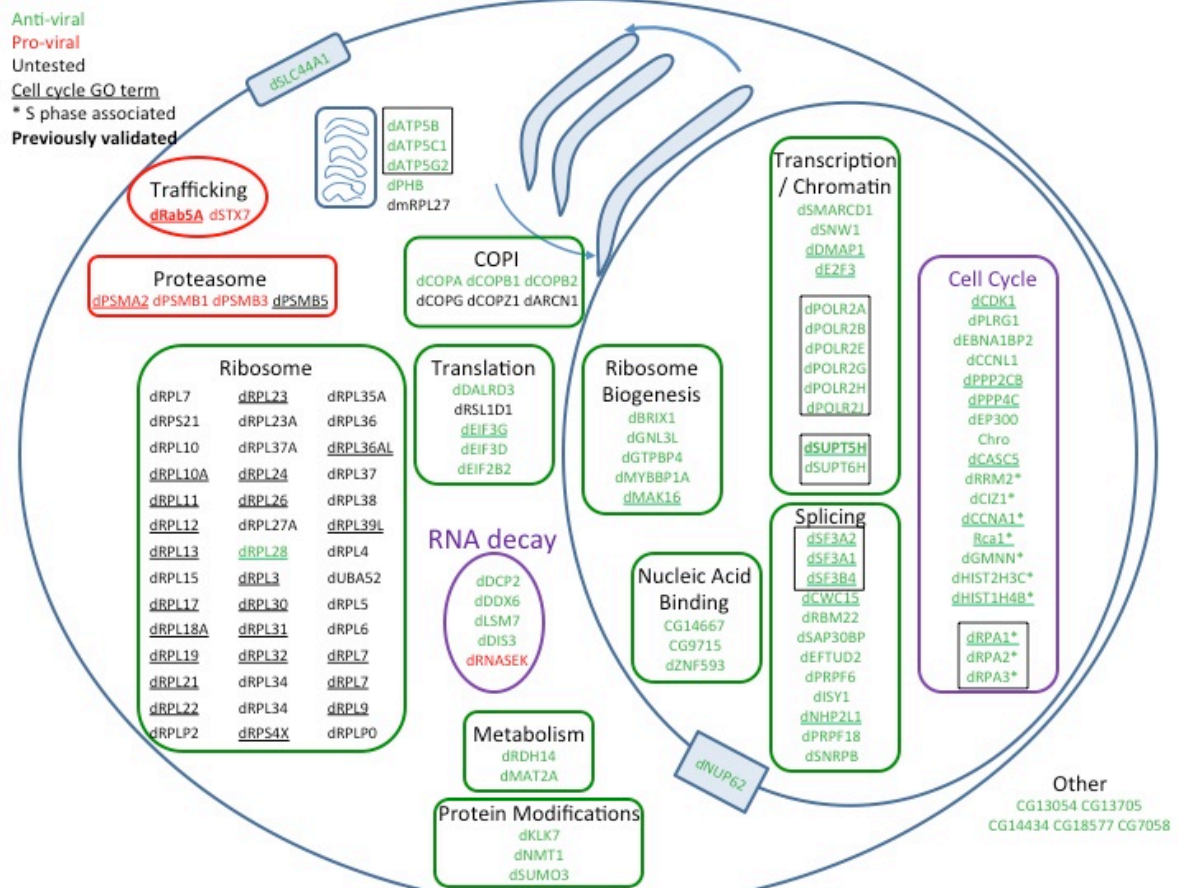


Altogether, we have explored the host factor dependencies of RVFV, leading to the finding that two diverse bunyaviruses are restricted by mRNA decapping. Furthermore, these studies have revealed new aspects of RNA decay and the regulation of these compartments; our results indicate that alteration of P body morphology during cell cycle progression is a deeply conserved process from insects to mammals (145). We also provide evidence that in *Drosophila*, Dcp2 decaps mRNAs involved in cell cycle progression and DNA replication, in addition to previously established roles in regulating histone mRNA levels in human cells (92). This suggests that decapping in *Drosophila* more generally targets mRNAs undergoing rapid turnover. RNA profiling studies in murine cells depleted of Dcp2 or NUDT16 suggest that in higher organisms the decapping of mRNAs is specialized (75). Thus, selective activation of decapping may potentially be a viable therapeutic approach to degrade RVFV accessible mRNAs. Indeed, Dcp2 is potentially regulated: in yeast, phosphorylation of Dcp2 by Ste20 is necessary for Dcp2 recruitment to P bodies (146), and vertebrate Dcp2 has a number of conserved uncharacterized phosphorylation sites (62). Further studies will reveal whether decapping of specific cargo can be selectively regulated in insects and mammals.

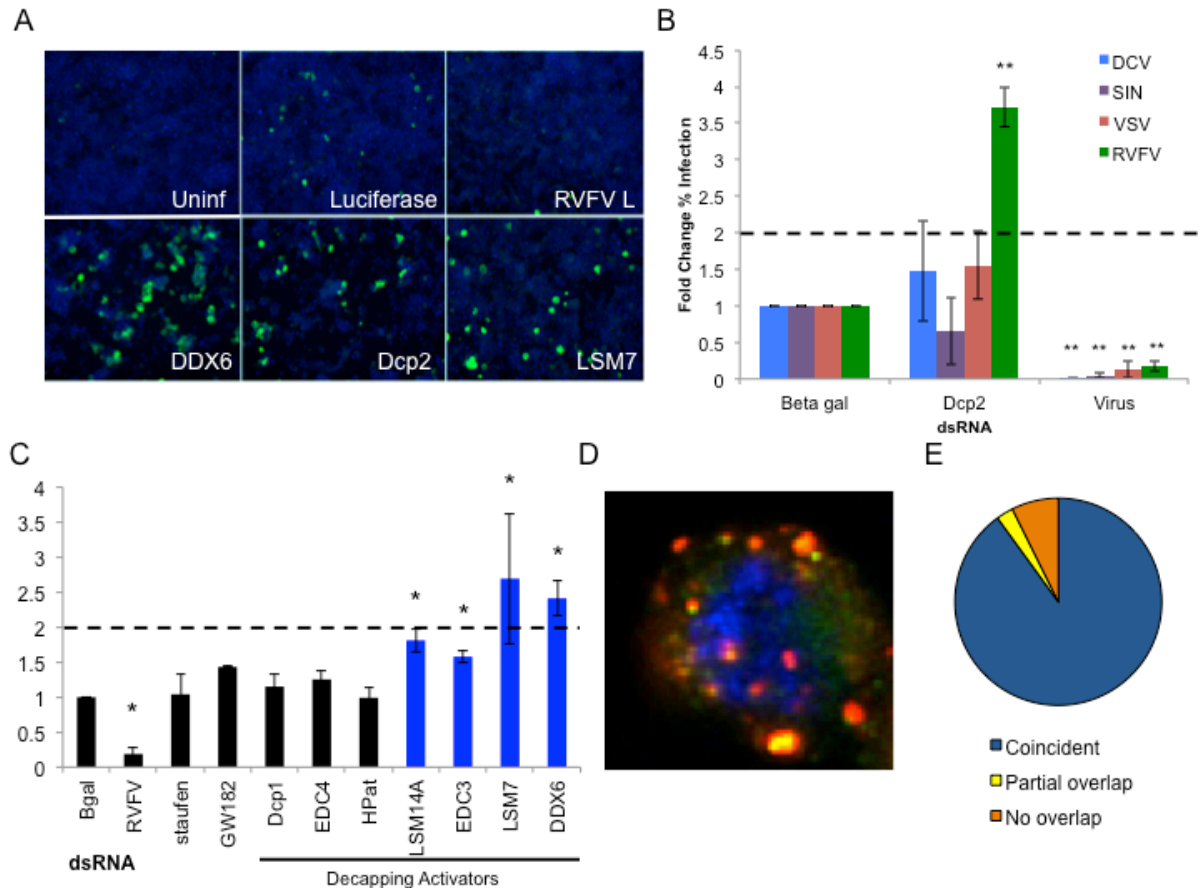


**Figure 3.1. Genome-wide RNAi screen for host factors that impact RVFV in *Drosophila*.**

**(A)** Genome-wide RNAi screen pipeline. Cells were plated onto 384 well plates pre-plated with dsRNAs targeting the *Drosophila* genome. Three days later cells were infected with RVFV MP12 (MOI=0.25) for 30 hours and processed for immunofluorescence (RVFV N, green; nuclei, blue). Automated microscopy followed by image analysis was used to calculate Robust Z-scores, which are shown for each replicate of the screen. **(B)** Primary candidates were validated using independent dsRNAs; for complexes with multiple candidates represented in the primary pool, a selection of genes were validated as representative of the complex. Robust Z-scores from 143 genes shown with validated genes in blue, genes that did not validate in grey. Dcp2, DDX6 and LSM7 are shown in red, green and orange, respectively. **(C)** GO term enrichment for validated genes.

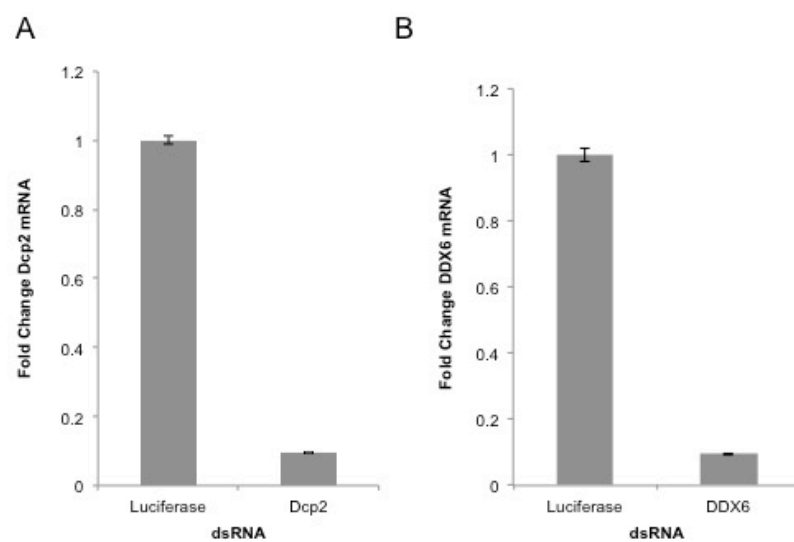


**Figure 3.2. Validated screen hits assigned to cellular functions based on informatics analysis.** Human gene names are shown for all orthologs. Genes in red are pro-viral, green are antiviral and black are members of large complexes not directly tested in secondary validation. Rab5C and SUPT5H were previously validated and are bolded (45, 143). Underlined genes have the GO term “cell cycle,” and an asterisk indicates S phase associated genes in the literature.

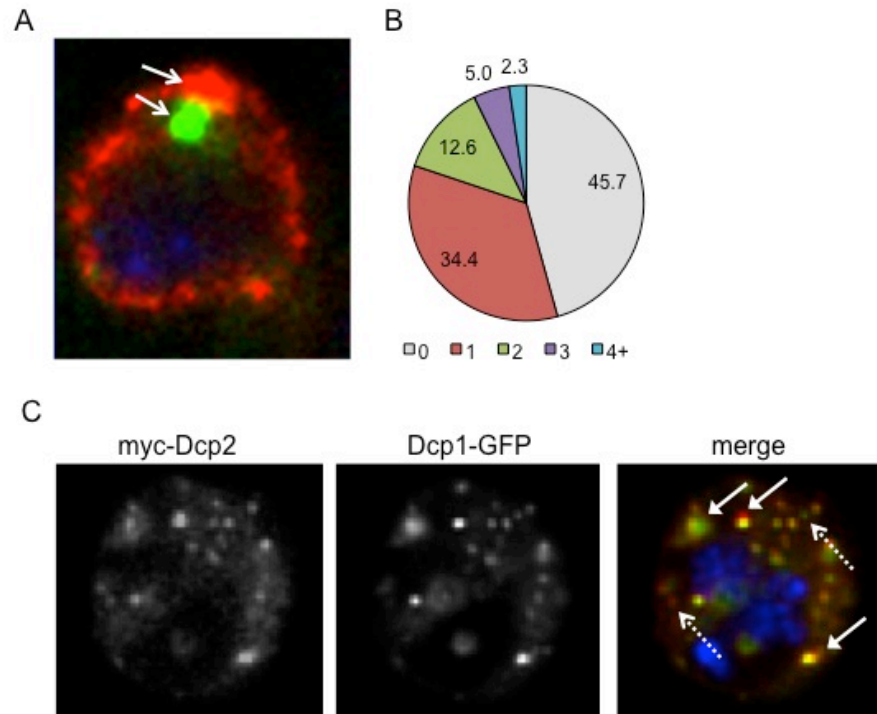


**Figure 3.3. Decapping restricts RVFV replication in *Drosophila* cells.**

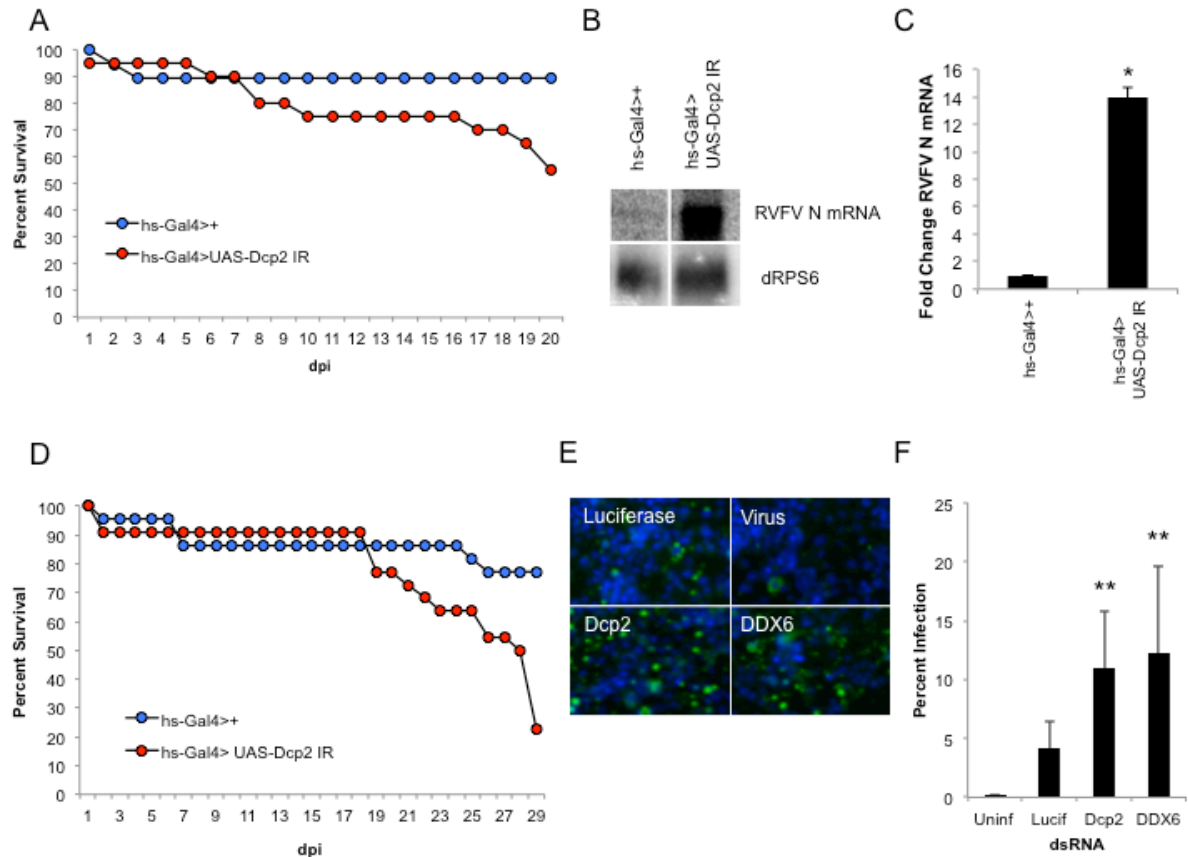
**(A)** *Drosophila* cells were treated with the indicated dsRNA for 3 days, then infected with RVFV (MOI 0.01) and processed for immunofluorescence (RVFV N protein, green; nuclei, blue). **(B)** Cells were treated as in **A** and infected with the indicated virus. dsRNA targeting each virus was used as a positive control (Virus). Quantification of mean fold change in the percentage of cells infected with the indicated virus is shown. Blue – *Drosophila* C Virus (DCV), purple – Sindbis virus (SIN), red – Vesicular Stomatitis virus (VSV), green – RVFV (MP12 strain). Mean±SD of ≥3 independent experiments; \*\*p<0.01. **(C)** Cells treated as in **A** with P body component dsRNAs were quantified for the mean fold change in the percentage of RVFV infected cells. Decapping activators are indicated, and genes significantly affecting RVFV replication are highlighted in blue. Mean±SD of ≥3 independent experiments; \*p<0.05. **(D)** Representative deconvolved plane of a *Drosophila* cell expressing myc-Dcp2 (green) and infected with RVFV for 30 hours (RVFV N red, nuclei blue). **(E)** Quantification of RVFV N and myc-Dcp2 punctae colocalization events per cell (>150 cells from 3 independent experiments). The majority of infected cells (~90%) have at least one colocalization between Dcp2 and RVFV N.



**Figure 3.4. Knockdown efficiency of dsRNAs.** Quantitative RT-PCR analysis of Dcp2 (**A**) and DDX6 (**B**) in cells treated with the indicated dsRNA normalized to RP49 levels and shown relative to non-targeting control (luciferase).

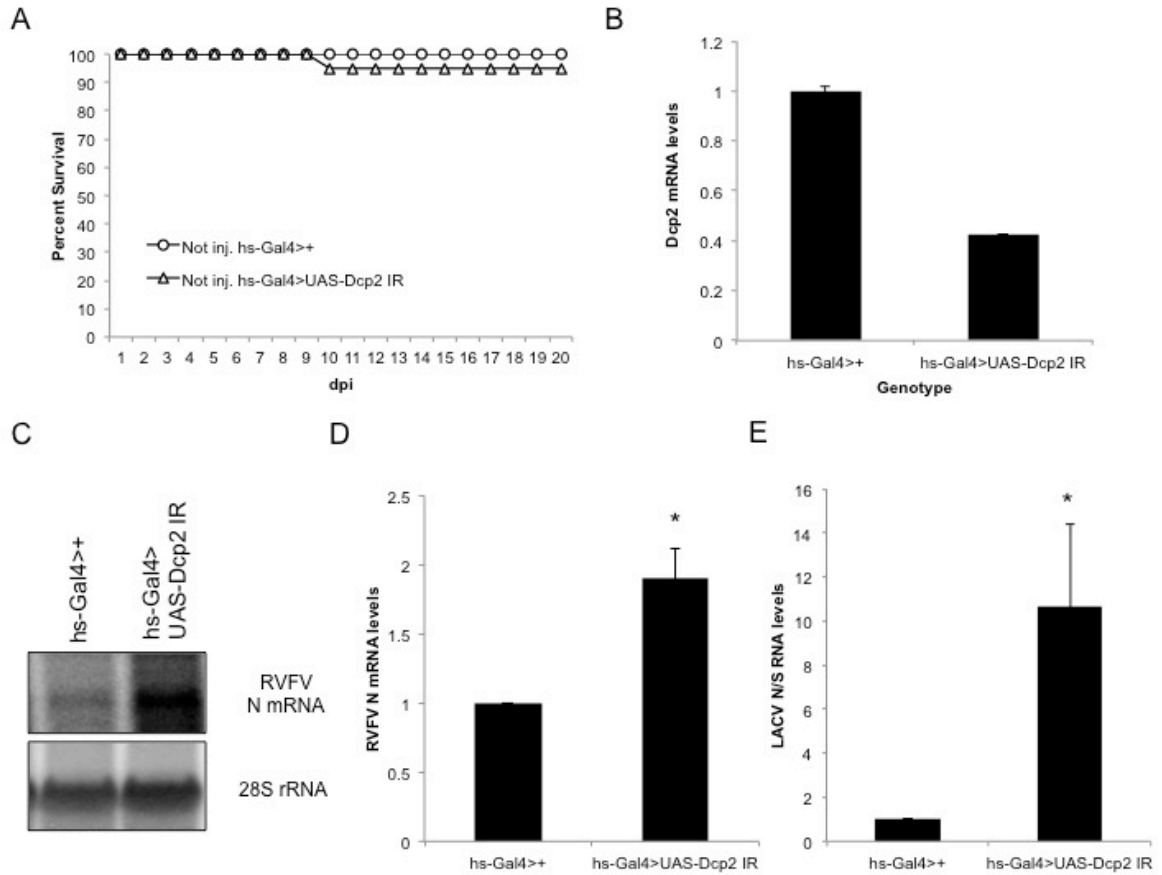


**Figure 3.5. Colocalization of Dcp1 with RVFV and Dcp2.** **(A)** Representative deconvolved plane of a *Drosophila* cell expressing Dcp1-GFP (green) and infected with RVFV for 30 hours (RVFV N red, total nuclei blue). **(B)** Quantification of RVFV N and Dcp1-GFP punctae colocalization events per cell (>300 cells from 3 independent experiments). The majority of infected cells (54.3%) have at least one colocalization between Dcp1 and RVFV N, although they are not coincident. **(C)** Representative deconvolved plane of a *Drosophila* cell expressing myc-Dcp2 (red) and Dcp1-GFP (green). Total nuclei stained in blue. The majority of punctae are both Dcp2 and Dcp1 positive with partial overlap shown as closed arrows. However there are some punctae that are only Dcp1 or Dcp2 positive shown as dotted arrows.



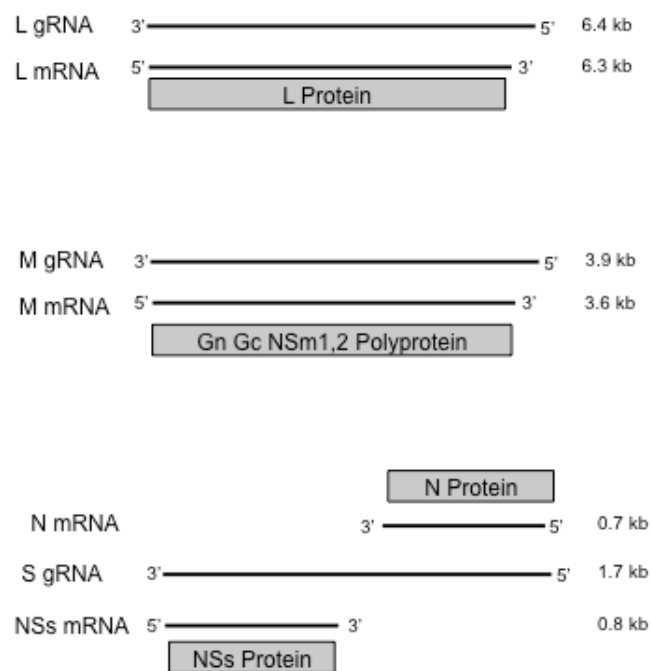
**Figure 3.6. Dcp2 restricts bunyaviruses in flies and mosquitoes.**

**(A)** Adult flies carrying heat shock inducible Gal4 were crossed to flies that inducibly express dsRNA against Dcp2 (hs-Gal4>UAS-Dcp2 IR, red) or controls (hs-Gal4>+, blue) were challenged with RVFV, and percent survival is graphed as a function of days post infection (dpi). A representative of at least three experiments is shown;  $p < 0.001$  log rank test. **(B)** Northern blot analysis of RVFV infected control flies and Dcp2-deficient flies probed for RVFV N mRNA or dRPS6 (cellular loading control) 6 days post infection. **(C)** Quantification of 3 experiments as shown in **B**. Mean $\pm$ SD; \* $p < 0.05$ . **(D)** Flies as described in **A** were challenged with La Crosse virus (LACV). Percent survival is graphed as a function of time. A representative of at least three experiments is shown;  $p < 0.001$  log rank test. **(E)** Representative immunofluorescence of *Aedes aegypti* Aag-2 cells treated with the indicated dsRNA and subsequently infected with RVFV. **(F)** Quantification of mean fold change in percent infection with mean $\pm$ SD shown; \*\* $p < 0.01$ .

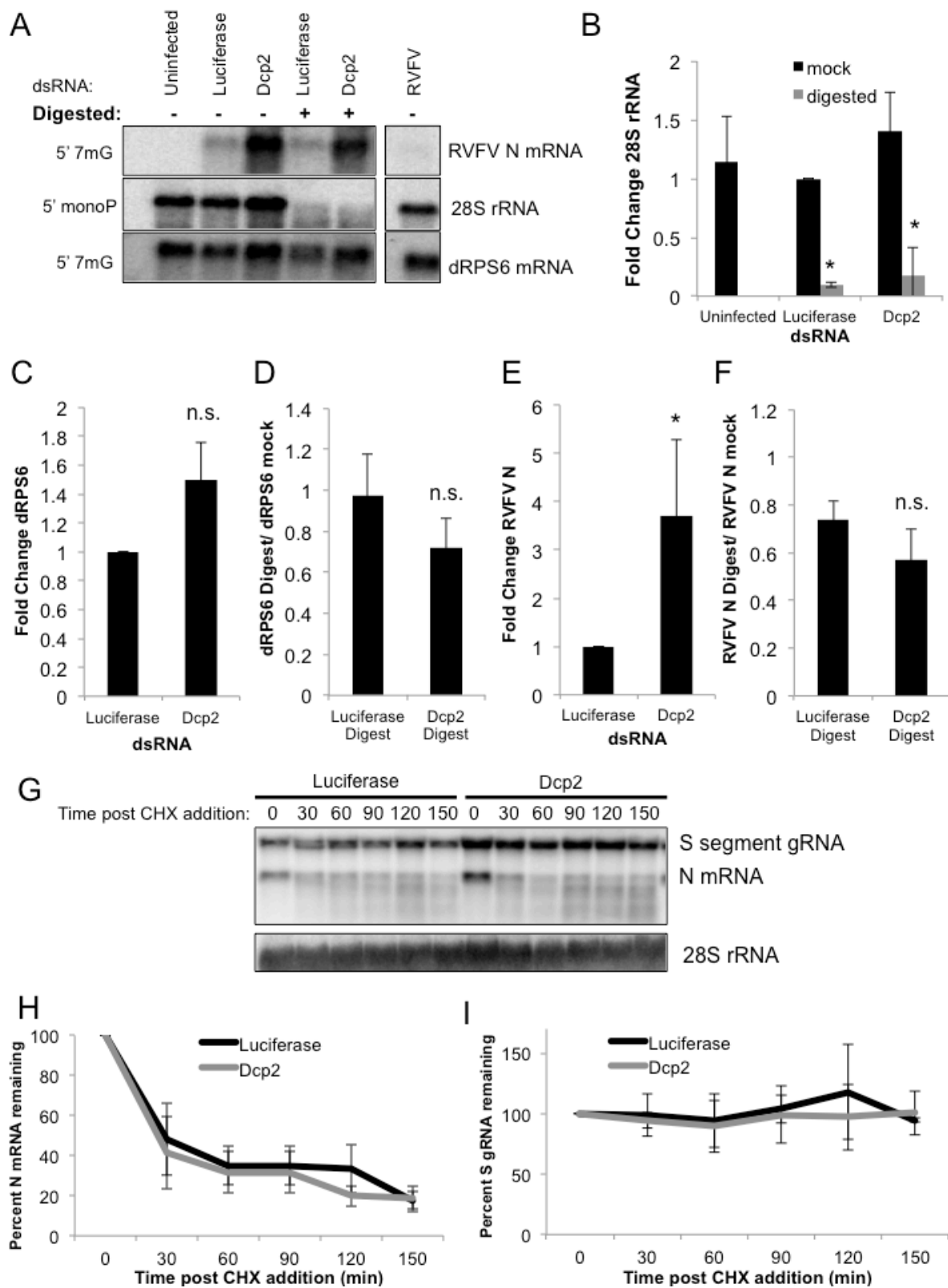


**Figure 3.7. Supplemental information for whole animal experiments. (A)** Survival of uninfected flies of the indicated genotypes. **(B)** Quantitative RT-PCR analysis of Dcp2 in adult heat shocked flies normalized to RP49 levels and shown relative to control flies. **(C)** Northern blot analysis of RVFV infected control flies and Dcp2-deficient flies probed for RVFV N mRNA or dRPS6 (cellular loading control) 20 days post infection. **(D)** Quantification of 3 experiments as shown in **C**. Mean $\pm$ SD; \* $p$ <0.05. **(E)** Quantitative RT-PCR analysis of LACV N/S RNA levels normalized to RP49 in infected flies of the indicated genotypes day 6 post infection. Mean $\pm$ SD for  $\geq 3$  independent experiments; \* $p$ <0.05.





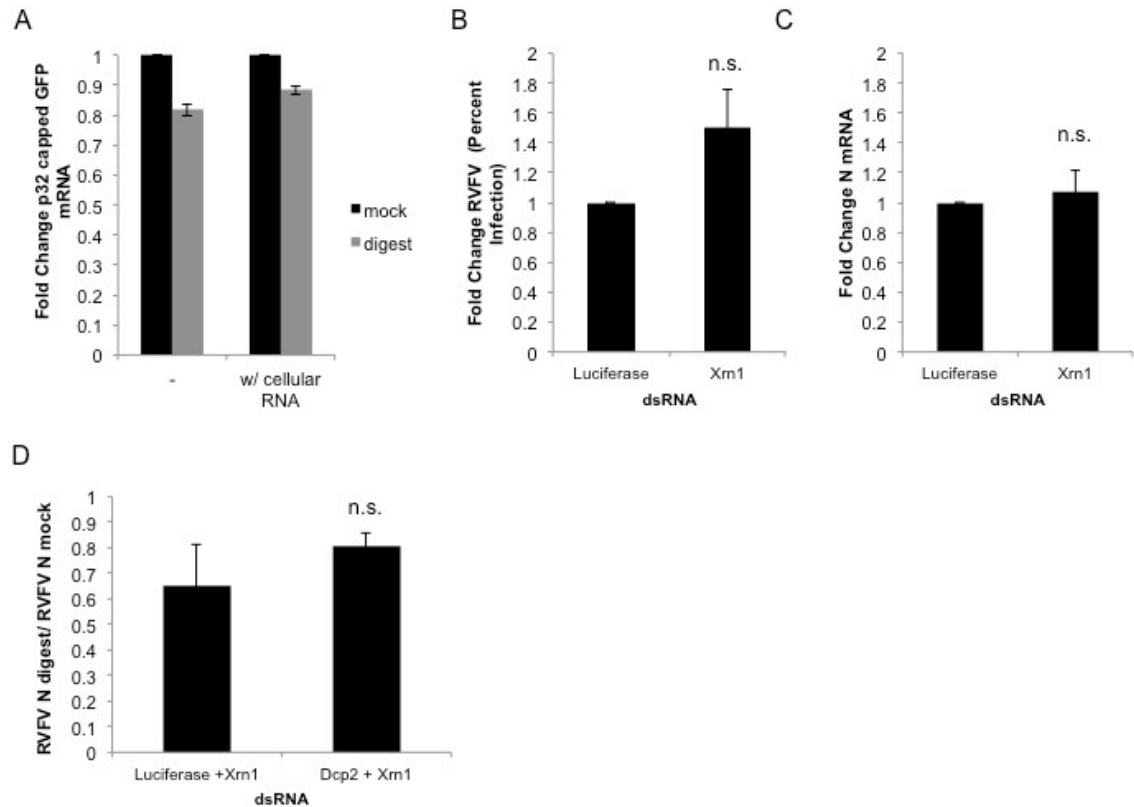
**Figure 3.8. Schematic of RVFV genomic RNA, mRNA and protein coding strategy.**



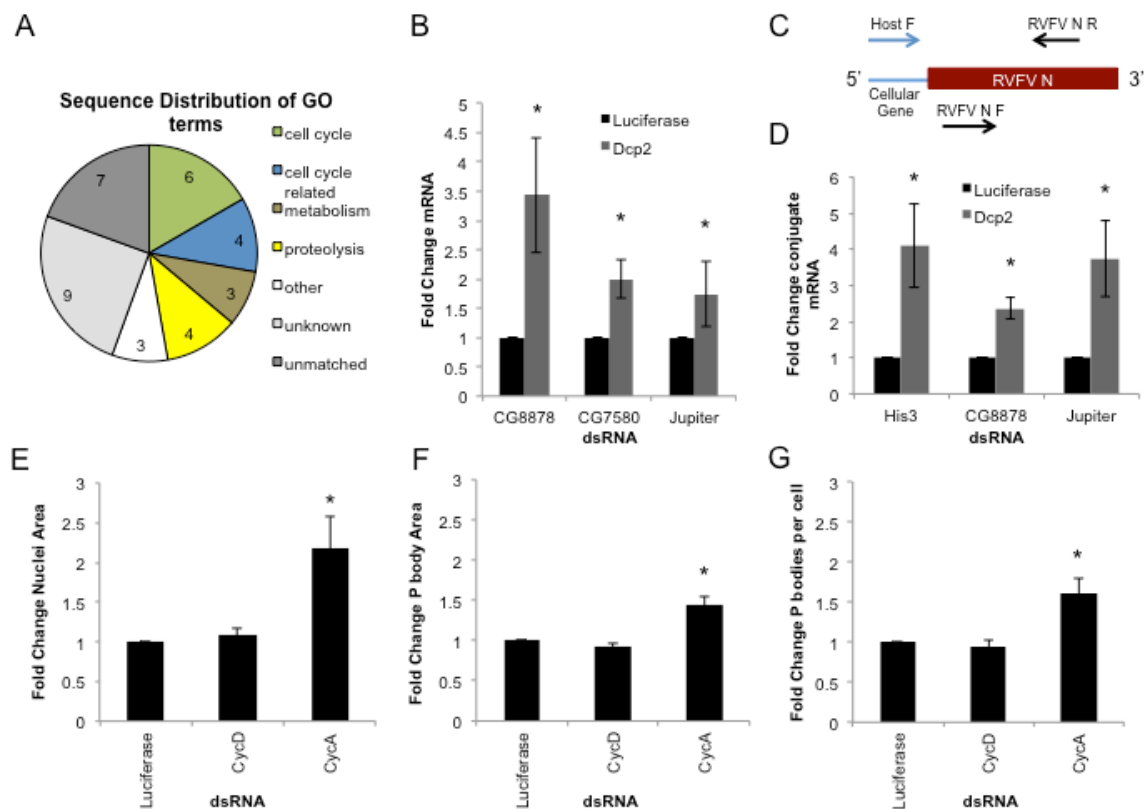
**Figure 3.9. Dcp2 does not directly degrade viral mRNA.**

(A) *Drosophila* cells treated with the indicated dsRNA and infected with RVFV; total RNA was either mock treated or digested with Terminator Exonuclease as indicated. Northern blots were

**Figure 3.9 (cont.):** probed for RVFV N mRNA (capped transcript), 28S rRNA (5' mono-phosphate bearing control transcript) or the cellular capped dRPS6 mRNA. **(B)** Quantification of 28S rRNA as shown in **A**, normalized to luciferase mock treated. Mean $\pm$ SD for  $\geq 3$  independent experiments; \* $p < 0.05$ . **(C)** Quantification of RVFV N mRNA in mock digest samples normalized to luciferase control. Mean $\pm$ SD for  $\geq 3$  independent experiments; \* $p < 0.05$ . **(D)** Ratio of digested RVFV N mRNA divided by the mock treated sample, yielding the ratio of mRNA that is refractory to digestion. Mean $\pm$ SD for  $\geq 3$  independent experiments; n.s. not significant. **(E)** Quantification of dRPS6 transcript in mock digest samples normalized to luciferase control. Mean $\pm$ SD for  $\geq 3$  independent experiments; n.s. not significant. **(F)** Ratio of digested dRPS6 mRNA transcript divided by the mock treated sample, yielding the ratio of mRNA that is refractory to digestion. Mean $\pm$ SD for  $\geq 3$  independent experiments; n.s. not significant. **(G)** *Drosophila* cells were treated with the indicated dsRNA and infected with RVFV. 28 hours post infection, cells were treated with cycloheximide (CHX), and total RNA was collected at the indicated time post CHX treatment. Northern blots were probed for RVFV N mRNA, the S segment genome, and 28S rRNA as a loading control. **(H)** Quantification of RVFV N transcript in CHX treated samples as shown in **G**. Mean $\pm$ SD for  $\geq 3$  independent experiments; no time points were significantly different between control and Dcp2-depleted cells. **(I)** Quantification of RVFV S segment genome in CHX treated samples as shown in **G**. Mean $\pm$ SD for  $\geq 3$  independent experiments; no time points were significantly different.



**Figure 3.10. Additional controls for Figure 3.9. (A)** GFP mRNA transcribed *in vitro* was capped with P-32 GTP, and digested or mock treated with terminator exonuclease in the presence or absence of *Drosophila* cellular RNA as indicated. RNA was run under denaturing conditions, transferred to a nitrocellulose membrane and radioactivity was quantified using ImageQuant. Mean of 2 experiments  $\pm$  SD. **(B)** *Drosophila* cells were treated with the indicated dsRNA for 3 days, then infected with RVFV (MOI 0.01) and processed for immunofluorescence. Percent infection was quantified and normalized to non-targeting control. Mean  $\pm$  SD of  $\geq 3$  independent experiments; n.s. = not significant. **(C)** Quantification of northern blot analysis from *Drosophila* cells treated with the indicated dsRNA and infected with RVFV. Northern blots were probed for RVFV N and normalized to 28S rRNA levels. Mean  $\pm$  SD of  $\geq 3$  independent experiments; n.s. = not significant. **(D)** DL1 cells were pretreated with the indicated dsRNAs, infected with RVFV and subject to the exonuclease assay. Northern blots were quantified and the ratio of digested RVFV N mRNA divided by the mock treated sample, yielding the ratio of mRNA that is refractory to digestion. Mean  $\pm$  SD for  $\geq 3$  independent experiments; n.s. not significant.



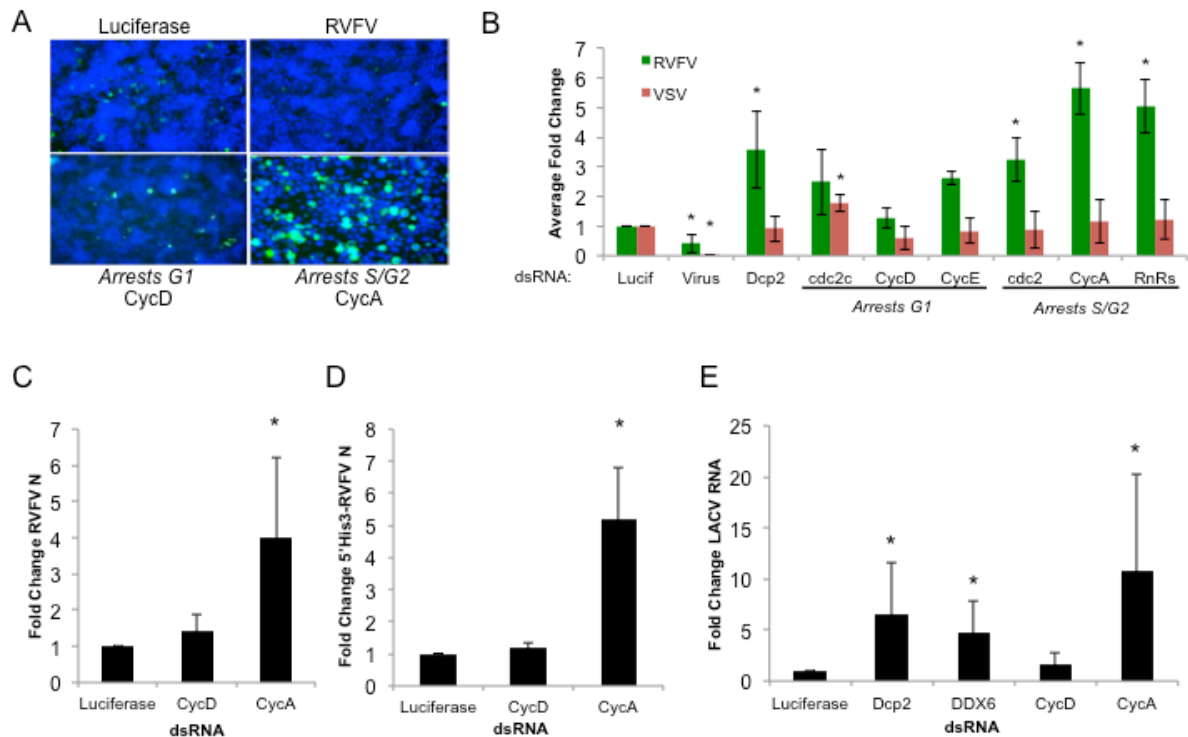
**Figure 3.11. Cell cycle RNAs are an enriched substrate for RVFV cap-snatching.**

**(A)** Pie chart of annotated GO function of sequences from the 5' end of RVFV mRNAs mapped to *Drosophila*. **(B)** Quantitative RT-PCR of levels of indicated target mRNAs, normalized to RP49 as a cellular loading control, and shown as fold change over non-targeting dsRNA (luciferase). Mean±SD for ≥3 independent experiments; \*p<0.05 **(C)** Schematic representing position of RT-PCR primers utilized in **D**. Forward primers recognizing the first 11 nucleotides of the host gene 5'UTR were used to amplify Host-RVFV fusion mRNA, and internal primers were used to amplify total RVFV N mRNA. **(D)** Quantitative RT-PCR of the indicated Host-RVFV N mRNA conjugates, normalized to RP49 as a loading control, and shown as fold change over non-targeting dsRNA (luciferase). Mean±SD for ≥3 independent experiments; \*p<0.05 **(E-G)** Cells expressing Dcp1-GFP, to monitor P bodies, were treated with the indicated dsRNA for 3 days, fixed, imaged and analyzed using MetaXpress software. Mean±SD for ≥3 independent experiments; n>500 cells; \*p<0.05. **(E)** Average nuclear area shown relative to luciferase control. **(F)** Average Dcp1-GFP foci size relative to luciferase control. **(G)** Average number of Dcp1-GFP foci per cell relative to luciferase control.

	Snatched sequence	Length (bp)	Match	Category
1	ATCTGCGCGTAGAC	14	14-3-3ζ	cell cycle
2a	CACGTTGCTCATGT	15	dp1	cell cycle
2b	CACGTTGCTCATGT	15	dp1	cell cycle
3a	AAAAACAGGAGAC	13	mars	cell cycle
3b	AAAAACAGGAGACG	14	mars	cell cycle
4	AAGAAAAAAGAAGACAC	17	pitslre	cell cycle
5	CTTTTCTTTTCC	12	RpS18	cell cycle
6a	ACACTACACAAAAAAAAC	18	SMF	cell cycle
6b	ACACTACACAAAAAAAAC	18	SMF	cell cycle
7	AGTTATAAATCAGG	14	CG15547	cell cycle related
8	ATTGTGTTTTTAC	13	Histone H3	cell cycle related
9	TTTAGCGGCTTAC	13	Jupiter	cell cycle related
10	AGTCTGAGTCTGAGAG	16	steppke	cell cycle related
11	ATTCGGCGACGC	12	FK506-bp2	chromatin related
12	ACTCTGTCACCGG	13	Dicer-2	gene regulation
13a	ACTGGTTGAGACG	13	CG7580	metabolism
13b	ACTGGTTGAGACG	13	CG7580	metabolism
14	ATTCGTGTTCC	11	CG7708	metabolism
15	AGAAGCAAATCAGCAC	16	Enolase	metabolism
16	AAAACTGCGTG	12	CG8878	phosphorylation
17	AGTTCATTTTTTG	13	CG17109	proteolysis
18	AGTCGTTAAGCTAGC	15	CG18557	proteolysis
19	AGTATGAATGCGCTGAT	17	CG33460	proteolysis
20	ACTTGGCCGCA(C)	13	Dipeptidase B	proteolysis
21	CTTTTACATCTT	13	CG 5375	unknown
22	AGTTTTTGTTT	11	CG10038	unknown
23	AGTTTCAAATAAA	13	CG11486	unknown
24	GTGAAAAGTACT	12	CG42542	unknown
25	(A)TTCACAACCAAATT	15	CG5103	unknown
26	AGTTCAACAGCTGTT	15	CG8297	unknown
27	ATTCGTTTTGAGCAG	16	cg9328	unknown
28	AGTTGTTTAACAATT	15	jvl	unknown
29	CGTCCATTACCAC	13	CR4378 (ncRNA)	unknown
30	CTTTTCGTTCTC	12	unmatched	
31	TTTTTGCTAGC	11	unmatched	
32	TTTTTCTGCCAGC	14	unmatched	
33	TTTTTTTTTTTTTTC	15	unmatched	
34	AGTTTTTTTTTTC	12	unmatched	
35	AGTTCATAGGCAC	13	unmatched	
36	AATAGAAAATTT	12	unmatched	

**Figure 3.12. Endogenous sequences cap-snatched by RVFV in *Drosophila*.** List of cap-snatched sequences, their length and corresponding *Drosophila* gene. GO terms are shown and

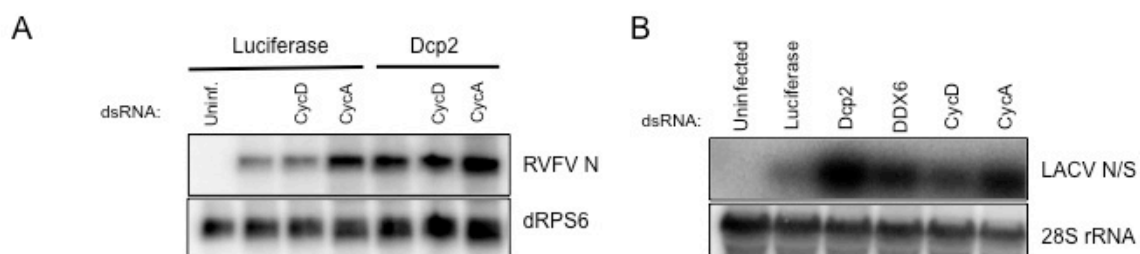
**Figure 3.12 (cont.):** color-coded to the pie chart in Figure 3.11A. Genes that were independently identified in multiple experiments are filled purple.



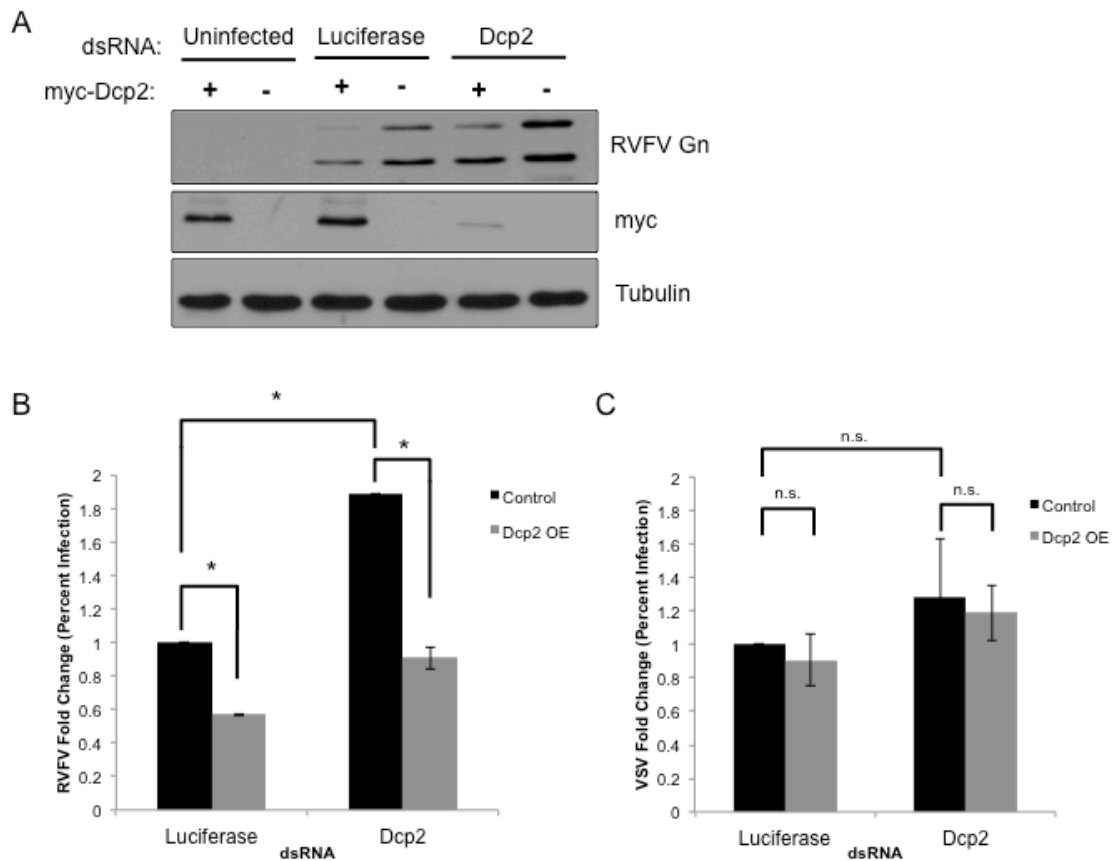
**Figure 3.13. Cell cycle arrest in late S/early G2 phase enhances bunyavirus replication.**

**(A)** Representative images of DL1 cells treated with the indicated dsRNA, infected with RVFV for 30 hours, fixed and stained for RVFV N protein (green) and total nuclei (blue). **(B)** Quantification of cells treated with the indicated dsRNA, then infected with either RVFV or VSV for 30 or 24 hours respectively. Mean $\pm$ SD for  $\geq 3$  experiments shown; \* $p < 0.05$ . **(C)** Cells were treated with the indicated cell cycle gene dsRNA. Quantification of northern blot analysis of RVFV N mRNA normalized to dRPS6 with the mean $\pm$ SD for  $\geq 3$  experiments shown; \* $p < 0.05$ . **(D)** Quantitative RT-PCR for 5'His3-RVFV N fusion mRNA in cells treated for the indicated dsRNA for 3 days, and then infected with RVFV for 30 hours. Mean $\pm$ SD for  $\geq 3$  experiments shown; \* $p < 0.05$ . **(E)** DL1 cells treated with the indicated dsRNA and infected with LACV for 72 hours. Quantification of northern blot analysis of LACV N mRNA and S segment genome/anti-genome normalized to dRPS6 with the mean $\pm$ SD for  $\geq 3$  experiments shown; \* $p < 0.05$ .





**Figure 3.14. Representative northern blots as in Figure 3.13. (A)** Representative northern blot of DL1 cells treated with the indicated dsRNAs and infected with RVFV for 30 hours quantified as in Figure 3.13C. **(B)** Representative northern blot of DL1 cells treated with the indicated dsRNA and infected with LACV for 72 hours quantified as in Figure 3.13E.



**Figure 3.15. Ectopic expression of Dcp2 restricts RVFV replication.**

**(A)** Control cells or cells expressing myc-Dcp2 were treated with the indicated dsRNA for 3 days, infected with RVFV for 30 hours and analyzed by immunoblot. **(B)** Cells were treated and infected as in **A**, processed for immunofluorescence and quantification of the percent infection with the mean $\pm$ SD for  $\geq 3$  experiments shown; \* $p < 0.05$ . **(C)** Cells were treated as described in **A** and infected with VSV for 24 hours. Cells were processed for microscopy and quantification of the percent infection with the mean $\pm$ SD for  $\geq 3$  experiments shown; n.s. not significant.

## **IV. THE MRNA DECAPPERS DCP2 AND NUDT16 LIMIT DISTINCT BUNYAVIRAL CAP-SNATCHING TARGETS TO RESTRICT INFECTION IN HUMANS**

### **1. Background**

Bunyaviruses are a genus of negative sense tripartite RNA viruses that infect diverse species, including plants and animals, and are largely vectored to vertebrate animals by arthropods, typically mosquitoes or ticks (16). Since these viruses have evolved to replicate in such disparate hosts, they likely hijack deeply conserved biological mechanisms to replicate. Previously, we found that two mosquito-transmitted bunyaviruses, the orthobunyavirus LaCrosse virus (LACV) and the phlebovirus Rift Valley Fever virus (RVFV), are restricted by the cellular RNA decapping machinery in insects (60). In a genome-wide RNAi screen to examine cellular factors impacting RVFV replication in *Drosophila*, we identified the canonical mRNA decapping enzyme, dDCP2, and two activators of decapping, Me31B (DDX6/Rck) and dLSM7, as antiviral against RVFV. Mechanistically, we found that mRNA decapping restricts the availability of cellular mRNA substrates used by these viruses for viral cap-snatching, a unique mechanism of viral mRNA transcription in which the 5' cap and 10-18 nucleotides (nt) of a host mRNA are used as an initiating primer for viral mRNA transcription. Moreover, we found that cell cycle mRNAs, which are under tight control by the decapping machinery, are the preferential targets used by RVFV, explaining the tight bottleneck imposed by dDCP2.

Since bunyaviruses also cap-snatch in vertebrates, we examined whether decapping is a conserved mechanisms of viral restriction in humans. While lower organisms from yeast to insects encode only one known decapper, DCP2, decapping is more complex in vertebrates (44). At least two mRNA decapping enzymes have

decapping activity in mice and humans, DCP2 and NUDT16 (117). DCP2 is the direct homolog of yeast and insect DCP2, while there are no clear homologs of NUDT16 in *Drosophila*. Recent studies have suggested that NUDT16 and DCP2 have both redundant and specific decapping targets, and they are thought to be downstream of specific RNA decay pathways (76). While microarray profiling has identified panels of DCP2 and NUDT16 sensitive targets in murine cells (76) and immunoprecipitation of human DCP2 has identified some RNA targets (78), the endogenous mRNA targets of human NUDT16 and what overlap they may possess with human DCP2 are largely unknown. Additionally, it is unclear how targets are differentially targeted to these enzymes and whether they share similar mechanisms of activation.

Since dDCP2 restricts RVFV in insects by decapping RVFV targets, we set out to determine if decapping also presents a bottleneck for RVFV replication in human cells. Indeed, we find that depletion of either DCP2 or NUDT16 led to increased RVFV replication, and enforced expression of either decapper also restricted viral replication. Since either loss of function or gain of function impact RVFV infection, these data suggest that DCP2 and NUDT16 are limiting in the cell. By sequencing snatched endogenous targets, we found that some targets were restricted specifically by either DCP2 or NUDT16, while others were restricted by both decappers. This suggests that RVFV cap-snatches in a niche where both DCP2 and NUDT16 are present. It is known that DCP2 resides in Processing (P) bodies, RNA-protein (RNP) complexes within the cytoplasm that contain the majority of RNA decay enzymes, including DCP2 and its known activators (44), and we previously showed that dDCP2 and the RVFV cap-snatching machinery colocalize in the cytoplasm. Minimal data regarding the localization of NUDT16 within the cytoplasm exists, and overexpression and endogenous antibodies have shown both punctate and diffuse cytoplasmic staining (81, 117). We find that in

addition to DCP2, NUDT16 is present in cellular granules indicative of P bodies. This indicates that RVFV cap-snatches in P bodies from DCP2 and NUDT16 targets. Intriguingly, we also find that RVFV infection leads to a loss of P body granules. This suggests that decapping may be activated as an antiviral mechanism to combat bunyaviral infection in humans. Overall, our data demonstrate a competition between RVFV cap-snatching and the cellular mRNA decapping machinery for the same pool of mRNA targets, and that these targets are limiting for viral replication. Furthermore, we identified bona fide targets of RVFV cap-snatching in humans, which are highly enriched for ribosomal proteins and other translationally related RNAs. Altogether, we demonstrate a conserved role for mRNA decapping in RVFV restriction and suggest that DCP2 and NUDT16 have both common and unique niches and targets that overlap with virally-targeted mRNAs.

## **2. Results**

### **Multiple mRNA decappers restrict Rift Valley Fever virus in human cells**

Since arboviruses, including bunyaviruses, replicate in Plants and in Animals from insects to humans (16), we set out to determine whether our finding that RVFV is restricted by dDCP2, the sole decapper in insects (61), is conserved in humans. We performed siRNA knockdown of the known mRNA decappers in humans, DCP2 and NUDT16 (118). While we only achieved modest knockdown at the mRNA level as measured by RT-qPCR (Figure 4.1A, B), we found that depletion of either DCP2 or NUDT16 lead to a significant increase in the relative infection of RVFV in U2OS cells, as measured by the percentage of infected cells (~2-fold; Figure 4.1C). DDX6/Rck is a known activator of DCP2-dependent decapping (35, 46) and restricts RVFV in insects

(61), and thus we also tested whether depletion of this factor impacted infection. Indeed, we found, similar to the effects seen with depletion of the decapping enzymes, a two-fold increase in the relative percentage of infected cells upon DDX6 depletion (Figure 4.1C). These data suggest that mRNA decapping is a broadly conserved pathway that restricts RVFV replication from insects to humans.

Our previous findings that decapping restricts bunyaviral replication at the level of RNA transcription in insects led us to examine whether RNA levels are increased in RVFV infected cells depleted of decapping enzymes. We found, similar to immunofluorescence studies, that total viral RNA levels are significantly increased upon depletion of DCP2 or NUDT16 as measured by RT-qPCR (~2-fold; Figure 4.1D). Since RT-qPCR does not distinguish the viral mRNA from the viral genomic RNA, future studies will examine the effects of knockdown of decapping enzymes by northern blot, as these RNA species can be distinguished based on size.

### **DCP2 and NUDT16 are limiting in human cells**

We set out to determine whether ectopic expression of either decapping enzyme could inhibit RVFV replication in humans. We established polyclonal stable cell lines expressing either Flag-tagged NUDT16 (118) or GFP-tagged DCP2 (126) and compared infection to control cells (Figure 4.2A, B). We observed low levels of these decappers after selection that was detectable only by an ultra-sensitive Enhanced Chemiluminescent (ECL) substrate capable of detecting protein in the low femtogram level by immunoblot (Thermo Scientific SuperSignal West Femto Substrate) (Figure 4.2C, data not shown), suggesting that abundant overexpression of decapping enzymes is deleterious in human cells.

Despite these low levels of protein expression, we observed significantly reduced levels of viral infection upon enforced expression of either DCP2 or NUDT16 compared to control cells as measured by multiple assays. First, we found that ectopic expression of DCP2 or NUDT16 significantly reduced the percentage of infected cells (Figure 4.2A, B). Second, we measured levels of viral glycoprotein, Gn, by immunoblot, and found that viral protein was significantly reduced in the presence of low-level enforced expression of NUDT16 (Figure 4.2C). Third, we found significantly reduced viral RNA by RT-qPCR with enforced NUDT16 expression (Figure 4.2D). This suggests that decapping is a highly regulated process, and that decappers are limiting in human cells. Future studies will examine the effects of enforced expression of DCP2 on viral protein and RNA.

### **Histone mRNAs are a conserved cap-snatching target of Rift Valley Fever virus**

We previously found that RVFV primarily snatches dDCP2 targets, which are largely cell-cycle-regulated mRNAs, for mRNA transcription, including replicating histone mRNAs (60). Therefore, we tested whether replicating histone mRNAs (Histone 3 and Histone 2A) are an endogenous target of RVFV cap-snatching in human cells. Thus, we used a PCR strategy to capture the specific host mRNA snatched by RVFV using with a forward primer against the first 11 nucleotides of the 5'UTR of our host gene of interest and a reverse primer against the viral N mRNA transcript. Using this strategy, we find that the histone mRNAs H2A and HIS3 are both targets of viral cap-snatching (Figure 4.3A, B). Interestingly, preliminary data show these target mRNAs appear to be increased upon depletion of either DCP2 or NUDT16, although NUDT16 appears to have a larger impact. Because either decapper impacts the levels of these mRNAs, our data suggests redundancy in target specificity between these decapping enzymes. This also suggests that decappers are limiting, since each enzyme is unable to fully

compensate for the loss of the other in degrading this pool of mRNAs. This also demonstrates for the first time that NUDT16 plays a role in the basal turnover of human histone mRNAs (92, 121).

Additionally, we examined whether enforced expression of NUDT16 reduces the levels of histone-viral mRNA conjugates. We found that both H2A- and His3-N conjugates were significantly reduced by low levels of NUDT16 ectopic expression (Figure 4.3C, D), similar to our observation of decreased total viral RNA (Figure 4.2D). These data suggest that NUDT16 levels are tightly regulated, and that minor alterations in their expression can profoundly affect the pools of transcripts used by bunyaviruses for replication. Future studies will examine the effects of enforced expression of DCP2 on these RNA species.

#### **RVFV primarily snatches translation related mRNAs, and these mRNAs are limited by RNA decapping**

We set out to determine the spectrum of mRNAs snatched by RVFV in human cells using 5'RLM-RACE (60). We obtained 83 sequences of non-viral origin from the 5' end of the viral N mRNA transcript (Table 4.1). The mean length of endogenously cap-snatched sequences was 13.1nt (S.D. +/- 1.35), and sequences ranged in length from 10-18 nt, consistent with previous reports (17). There was little sequence preference in the first 10nt of the snatched sequences, with the exception of a slight preference for a T at position 2 (Figure 4.4A). There was a strong preference for the last nucleotide of the snatched sequence before the initiation of the viral sequence to be a C or G (Figure 4.4B), and this was slightly influenced by the length of the sequence; sequences 12 or 15 nt in length had a strong C preference, while those 13-14 nt in length showed increased G preference for a terminal G residue (Figure 4.4C). Thus, there is a strong



bias for the RVFV polymerase to cleave endogenous host mRNAs following a residue that base pairs with three hydrogen bonds.

Interestingly, of the 49 sequences we were able to match back to annotated 5' UTRs in the human genome, we found a strong enrichment for genes involved in mRNA translation, including RNAs encoding protein components of the ribosome and translation initiation and elongation factors (Table 4.1) (Figure 4.4D). This is in contrast with our previous findings in insects, where we found that cell cycle related messages were preferentially incorporated into RVFV mRNAs (60). This suggests that specific cohorts of genes are targeted during viral cap-snatching, and that these RNAs vary between hosts. Potentially, these mRNAs may be in niches where cap-snatching and RNA degradation overlap; future work will examine basal levels of these transcripts upon perturbation of decapping to determine whether decappers are a driving force for translational mRNA stability.

Of the endogenous targets observed, we examined the incorporation of two ribosomal protein mRNAs, RPL37A and RPS3A, into viral mRNA conjugates. Similar to histone mRNAs, incorporation of the 5' end of RPL37A into viral mRNA was increased by either DCP2 or NUDT16 depletion, suggesting that this mRNA is redundantly degraded by these enzymes, but that in the absence of one, the other decapper is unable to compensate (Figure 4.4E). However, we found loss of NUDT16 more profoundly impacted incorporation than DCP2, suggesting potential target specificity. In contrast to the redundancy on histone mRNAs, we found that RPS3A-viral RNA conjugate levels were dramatically increased upon NUDT16 depletion, but were unaffected by DCP2 depletion (Figure 4.4F). This suggests that RPS3A is a specific target of NUDT16 decapping, and that it is stabilized upon NUDT16 depletion. Further studies will examine the basal levels of these targets in uninfected cells depleted of

decappers, and whether any of our other endogenously snatched targets are DCP2 specific in their manner of decay.

### **Rift Valley Fever virus infection triggers P body loss in human cells**

DCP2 and other RNA decay machinery reside in cytoplasmic P bodies. We found that RVFV nucleocapsid protein, N, co-localizes with dDCP2-containing P bodies in insect cells, and Mir et al. have demonstrated that the bunyavirus Sin Nombre N protein co-localizes with DCP1A-containing P bodies in human cells. Thus, we examined the interplay between RVFV N and P bodies in human cells. First, we visualized P bodies as a function of time post infection using the P body marker DDX6, which we have also shown is a conserved antiviral factor ((60) and Figure 4.1C). Surprisingly, we found that by 12 hours post infection, DDX6 positive punctae were lost in infected cells, but not in neighboring uninfected cells in the same field (Figure 4.5A, B). Since DDX6, but not DCP1A, can be found in related cytoplasmic RNPs called stress granules, we examined DCP1A punctae during RVFV infection. We found that DCP1A positive punctae were also lost during RVFV infection (Figure 4.5C, D).

The localization of NUDT16 in the cytoplasm has not been carefully studied; it is unclear whether NUDT16 resides in P bodies, where DCP2 and known decapping activators are present. Additionally, whether DCP2 and NUDT16 have overlapping locations in the cell has not been examined. Due to technical issues, we were unable to visualize endogenous DCP2 or NUDT16 localization in the cytoplasm to examine whether: 1) these proteins are punctate in P bodies; 2) whether these punctae colocalize with RVFV N; and 3) whether any punctae dissociate with RVFV infection. However, preliminary studies examining Flag-NUDT16 did show distinct punctate cytoplasmic staining, reminiscent of P bodies (Figure 4.5E). Future studies will clarify the localization

of Flag-NUDT16 with DCP2 and other known P body markers basally and in the context of RVFV infection.

### **P body depletion is not autophagy dependent**

One possible mechanism that can potentially account for the loss of P bodies is capture and degradation of these RNPs by autophagy (20). Autophagy is a highly conserved process in which large materials, such as damaged organelles, are captured by a double membrane structure termed an autophagosome, which then fuses with a lysosome that releases its lytic enzymes to complete the degradation of its contents (82). Stress granules are degraded through autophagy in a conserved manner from yeast to humans, and basal levels of P bodies are increased in autophagy deficient yeast (20). We set out to determine whether autophagy activation is responsible for the clearance of P bodies in RVFV infected cells, as this could explain P body loss.

We found that in U2OS cells treated with siRNAs against the essential autophagy proteins, ATG5 and ATG7, viral induced clearance of P body punctae was unaffected (Figure 4.6A, B). Furthermore, we used ATG5 knockout MEFs, which cannot undergo autophagy, and again observed no affect on P body clearance compared to wild type MEFs (Figure 4.6C, D). These data demonstrate that RVFV-induced P body clearance is independent of autophagy.

### **3. Discussion**

Bunyaviruses infect widely disparate organisms from plants to insects to humans. We previously found that dDCP2 creates a bottleneck of mRNA substrates for RVFV cap-snatching in *Drosophila* (60). Since RVFV also infects mammals, including humans, we set out to explore the role of decapping in RVFV infection in human cells. Recent

studies have revealed the increased complexity of mRNA decapping in mammals, and unlike yeast and *Drosophila*, where only one mRNA decapper is known, at least two decappers exist in mammals (118). Interestingly, the specificity of these decappers is only beginning to be unraveled (76). We found that decapping is a conserved mechanism by which cells restrict bunyaviruses. We found that depletion of either DCP2 or NUDT16 led to increased RVFV replication, demonstrating a lack of redundancy in their activity and an inability to fully compensate for loss of one another. This is in line with previous data showing that these enzymes may have both redundant and specific endogenous targets and functions within specialized RNA decay pathways (76). Indeed, we found that RVFV snatching of RPS3A mRNA is increased upon loss of NUDT16 but not DCP2, while RPL37A mRNA is increased upon loss of either DCP2 or NUDT16. This demonstrates both specific and overlapping dependencies.

While it was known that DCP2 resides in P bodies from yeast to humans, the localization of NUDT16 within the cytoplasm was unknown. We found that these decappers reside in the same compartment where RVFV N is targeting mRNAs. Preliminary data suggest that in the context of enforced expression, Flag-NUDT16 forms cytoplasmic puncta (Figure 4.5E); these may be indicative of P bodies, and further work will examine the spatial localization of NUDT16 in relation to other P body components. As we and others have previously found that P bodies are not of uniform composition (111, 141), even among the binding partners dDCP2 and dDCP1A in insects (61), the possibility exists that NUDT16 and DCP2 specificity and redundancy could be explained by differential targeting of RNAs to P bodies containing individual or both decappers. While mechanistically it is unknown how specific mRNAs are targeted, it is possible that decapping activators, which are known to bind RNA, provide some level of specificity. These may either bring mRNAs to P bodies in general or perhaps to specific decappers.

Since little is known about NUDT16, and it is not conserved in yeast where most decapping activators were first identified, it is possible that there exist decapping activators that specifically impact NUDT16 function. Future work will examine the composition of these compartments to attempt to understand how RNAs are targeted to them. Indeed, there may be additional uncharacterized decappers in mammals. A recent report by Song et al. using *in vitro* decapping assays identified six additional NUDIX domain containing proteins in mice with decapping activity (119).

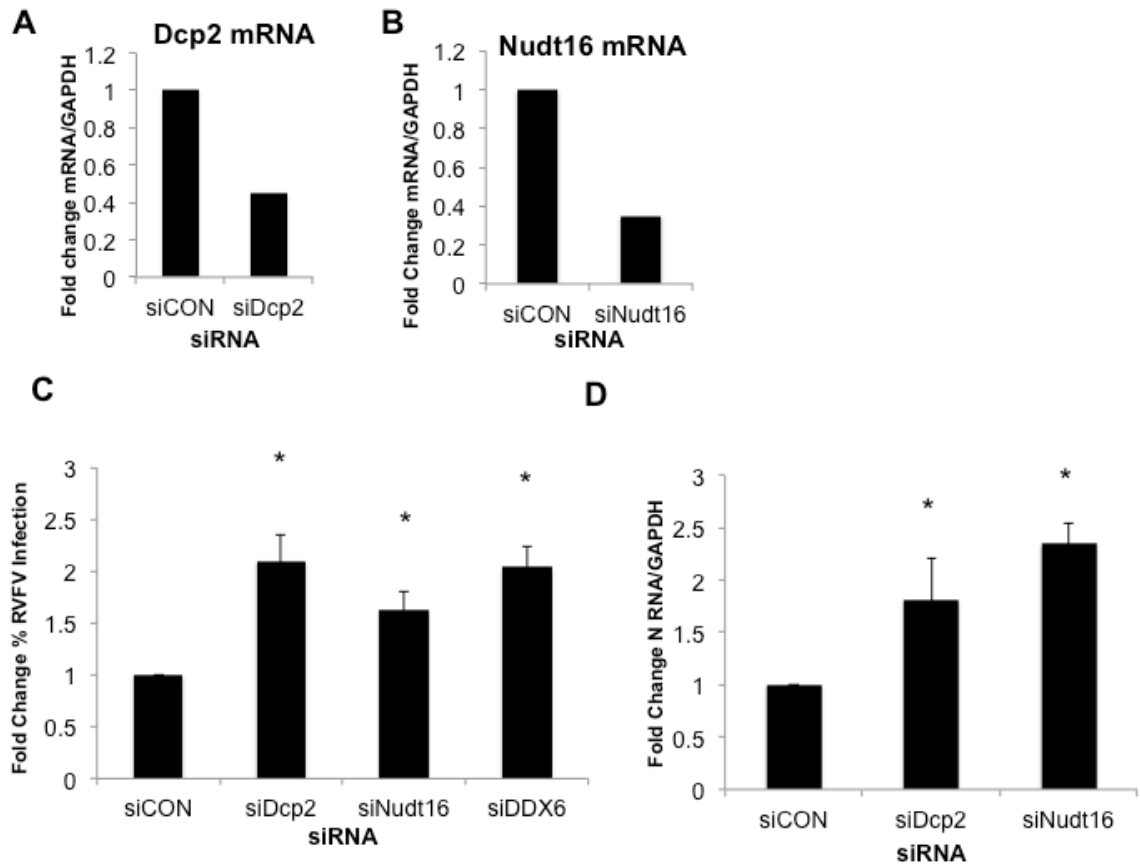
We found that RVFV preferentially cap-snatches mRNAs associated with translation and that the decappers restrict the incorporation of these mRNAs into viral transcripts. Indeed, we found seven ribosomal protein mRNAs, one translation initiation factor and one elongation factor. This suggests that this cohort of functionally related mRNAs is coordinately regulated post-transcriptionally by decapping. RNA operons have been defined as pools of functionally related genes that are coordinately regulated post-transcriptionally by sequence specific RNA binding proteins. While some RNA operons are well defined (e.g. histone mRNA stability regulated by stem loop binding protein), others have only suggestive evidence (69). Intriguingly, some of the best evidence for the coordinated regulation of functionally related genes at the level of mRNA stability comes from studies of immune-related gene programs. The mammalian RNA binding proteins ELAV/Hu and TTP target chemokine and cytokine mRNAs to promote their stability, and the half-lives of these RNAs are synchronously altered during immune responses (69). Other evidence for functionally coordinated decay operons comes from microarray data examining the half-lives of RNA transcripts in yeast; functionally related genes, especially those within the same complex, have similar half-lives (137). Indeed, one of the complexes demonstrating a high degree coordination in the decay rate of its components is the ribosome (137). This suggests that the directed decay of translation

related mRNAs is deeply conserved. Overall, these findings imply that decapping activation could be a useful tool to regulate RNAs through targeted RNA decay, or that inhibition of decapping could dramatically stabilize these programs.

Our findings that translation related mRNAs are preferentially snatched during RVFV infection are intriguing; if cap-snatching and RNA decapping are indeed competing processes, this suggests a high level of targeted degradation of these translational mRNAs. mRNAs encoding the core translational machinery, such as ribosomal proteins, are known to have 5'Terminal Oligopyrimidine (TOP) motifs, consisting of cytosine- and uridine-rich stretches greater than 4 bases at the extreme 5' end of the mRNA (5). The translation and polysome occupancy of TOP motif containing mRNAs has been shown to be coordinately regulated in response to mTOR inhibition (123). We hypothesize that upon RVFV infection, translation related mRNAs are specifically targeted for degradation by decapping as a means to restrict translation of viral RNAs to combat infection within the cell. Indeed, many known antiviral programs shutdown translation (37, 68). PKR inhibits translation through the phosphorylation of EIF2A, but its activity is blocked by RVFV (63). IFIT proteins are interferon-induced and inhibit translation initiation at multiple steps, however, RVFV does not induce interferon efficiently (10). Additionally, as bunyaviruses have evolved an encoded ability to recruit ribosomes without the aid of the EIF4F complex and can recruit the 43S pre-initiation complex via their nucleocapsid protein (84), this suggests ancient evolutionary pressure to find novel means of ribosome recruitment and translation. As these viruses steal endogenous caps, which should not stimulate an immune response, the need to develop this activity is not obvious. DCP2 is potentially inducible by interferons (109) in humans and by poly I:C and lentiviral infection in murine cells (75). Thus, decapping of translation mRNAs may potentially be a novel innate immune effector response. Future work will

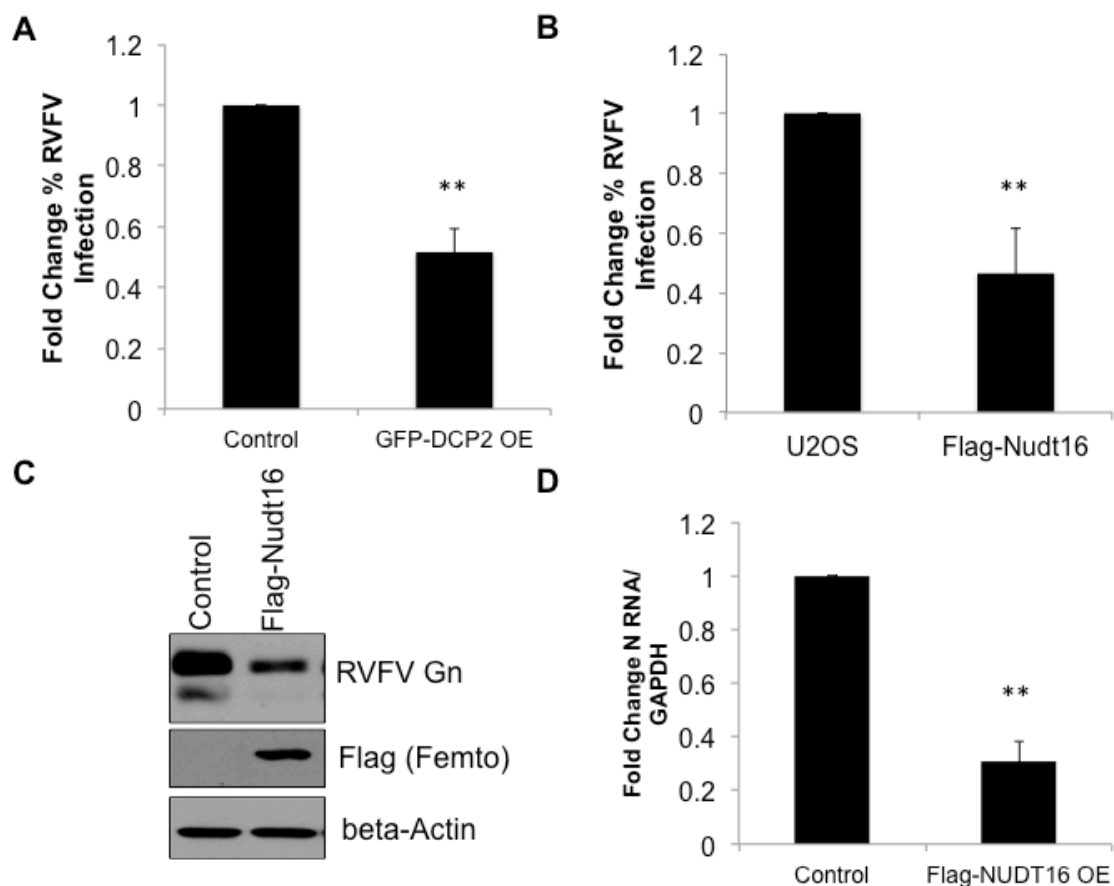
examine whether the stability of these mRNA targets is affected by perturbation of decapping basally in uninfected cells, to see if this targeted degradation is indeed triggered as a host response to viral infection.

Since only subtle increases in the levels of decappers are sufficient to attenuate RVFV replication, this suggests that decapping activation may be a reasonable therapeutic target to restrict bunyaviral infection. The potential to harness decapping as an antiviral mechanism or as a tool to control the expression of specific cohorts of genes is exciting. As theoretically druggable targets, decapping activation could be a novel mechanism for targeted gene regulation in multiple contexts.

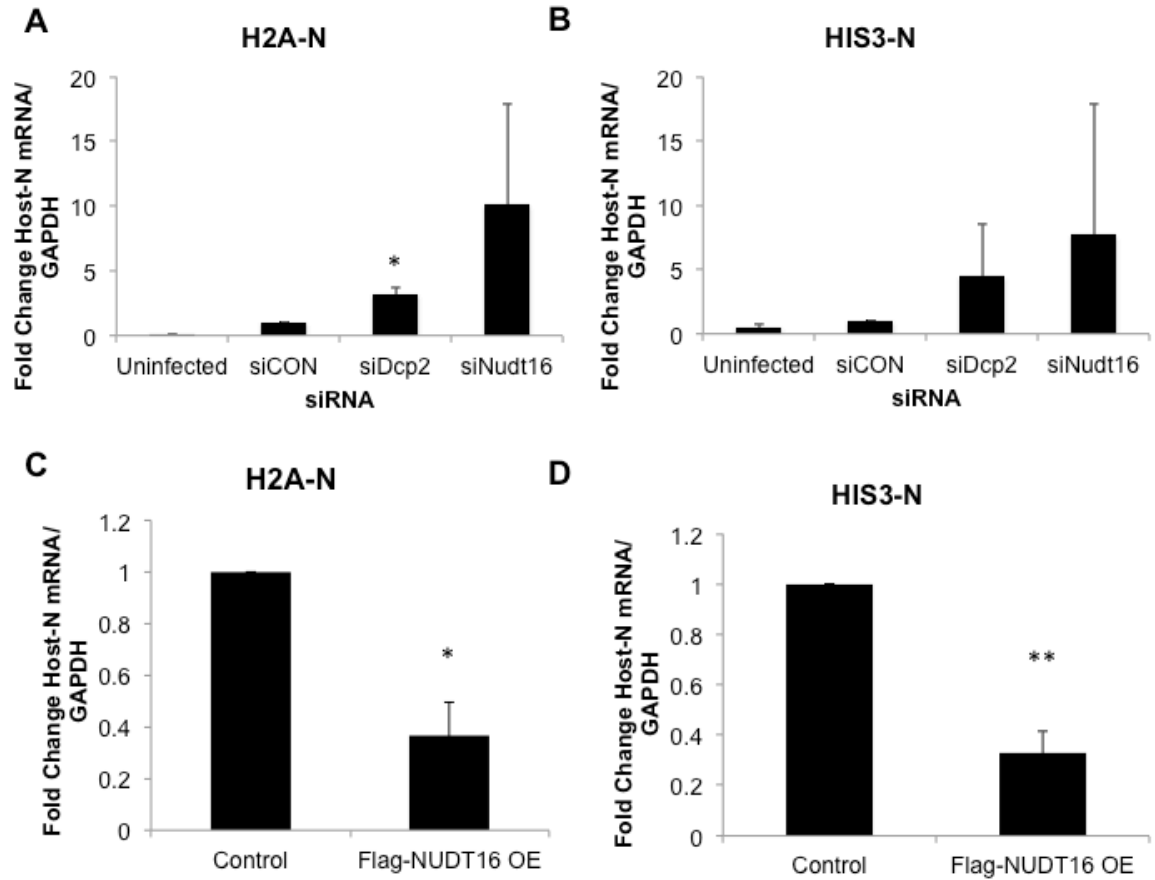


**Figure 4.1. RVFV replication is increased by depletion of human mRNA decapping enzymes in U2OS cells.** (A) RNA collected from U2OS cells treated with two pooled siRNAs targeting DCP2 three days post transfection was analyzed by RT-qPCR for knockdown efficiency. Fold change is normalized to GAPDH (n=1). (B) Cells were treated as in A with siRNAs targeting NUDT16 and analyzed by RT-qPCR for knockdown efficiency. Fold change is normalized to GAPDH (n=1). (C) U2OS cells were treated with the indicated siRNAs and then infected with RVFV (strain MP12, MOI=0.4) for 18 hours. Cells were fixed and stained for total nuclei and RVFV N protein by immunofluorescence. Automated image analysis was used to determine the percentage of infected cells. Relative infection is shown (n=3, \*p<0.05). (D) U2OS cells were treated with the indicated siRNAs and then infected with RVFV (MOI=1). Total RNA was collected 20hpi and analyzed by RT-qPCR for RVFV N RNA. Fold change is normalized to GAPDH (n=3, \*p<0.05).



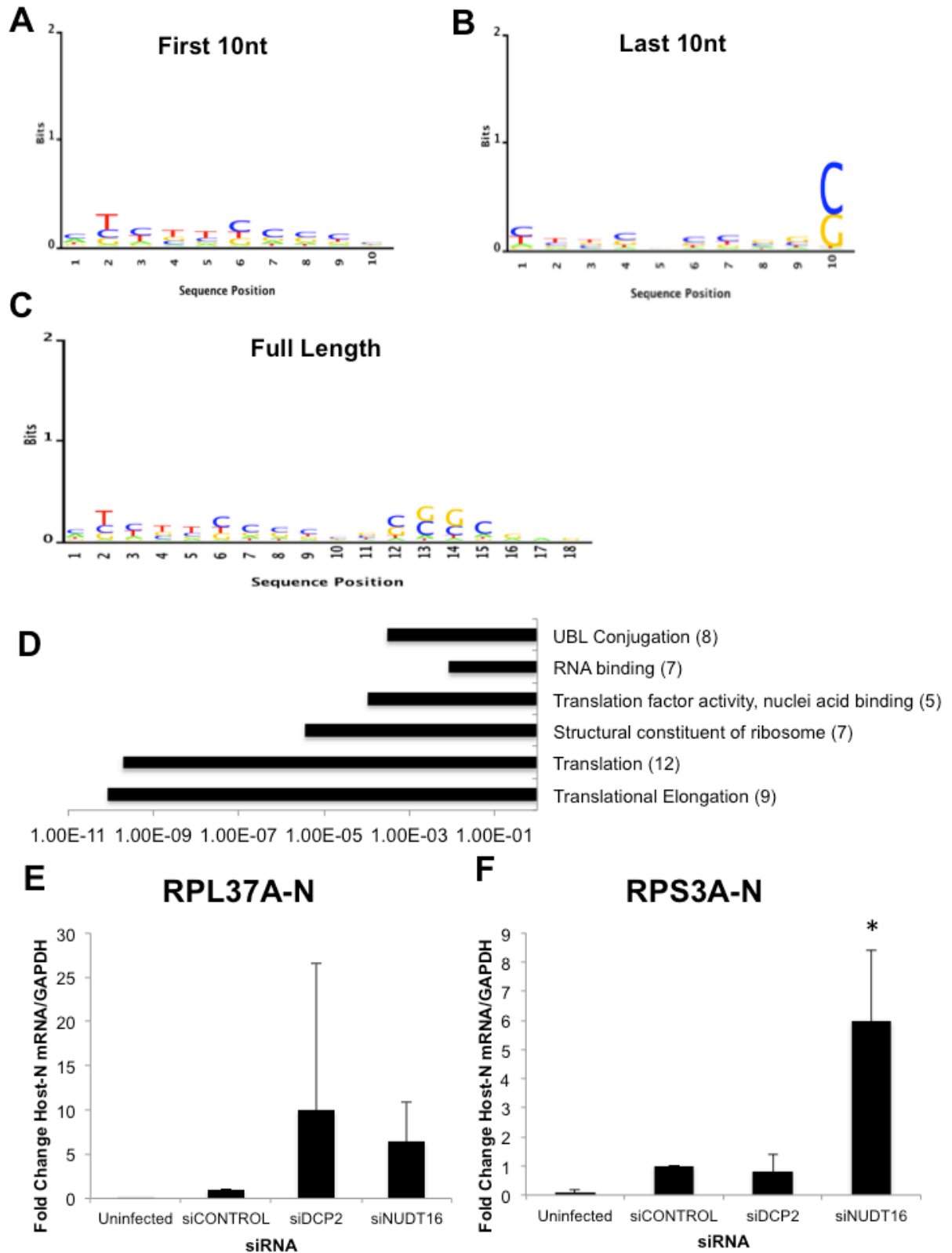


**Figure 4.2. mRNA decapping enzymes are limiting for RVFV infection in humans.** (A) U2OS cells or stable U2OS cell lines overexpressing GFP-tagged DCP2 were infected with RVFV (MOI=0.4). Cells were fixed and stained for total nuclei and RVFV N protein by immunofluorescence. Automated image analysis was used to determine the percentage of infected cells. Relative infection normalized to control cells is shown (n=3, \*\*p<0.01). (Figure 4.1A, B) U2OS cells or stable U2OS cell lines overexpressing Flag-tagged NUDT16 were infected with RVFV (MOI=0.4). Cells were processed as in **A**. Relative infection normalized to control cells is shown (n=3, \*\*p<0.01). (C) Control cells or cells expressing Flag-tagged NUDT16 were infected with RVFV (MOI=1) and total protein was collected 20 hpi and analyzed by immunoblot. A representative of three experiments is shown. (D) Control cells or cells expressing Flag-tagged NUDT16 were infected with RVFV (MOI=1) and total RNA was collected 20 hpi and analyzed by RT-qPCR for RVFV N RNA. Fold change is normalized to GAPDH (n=3, \*\*p<0.01).



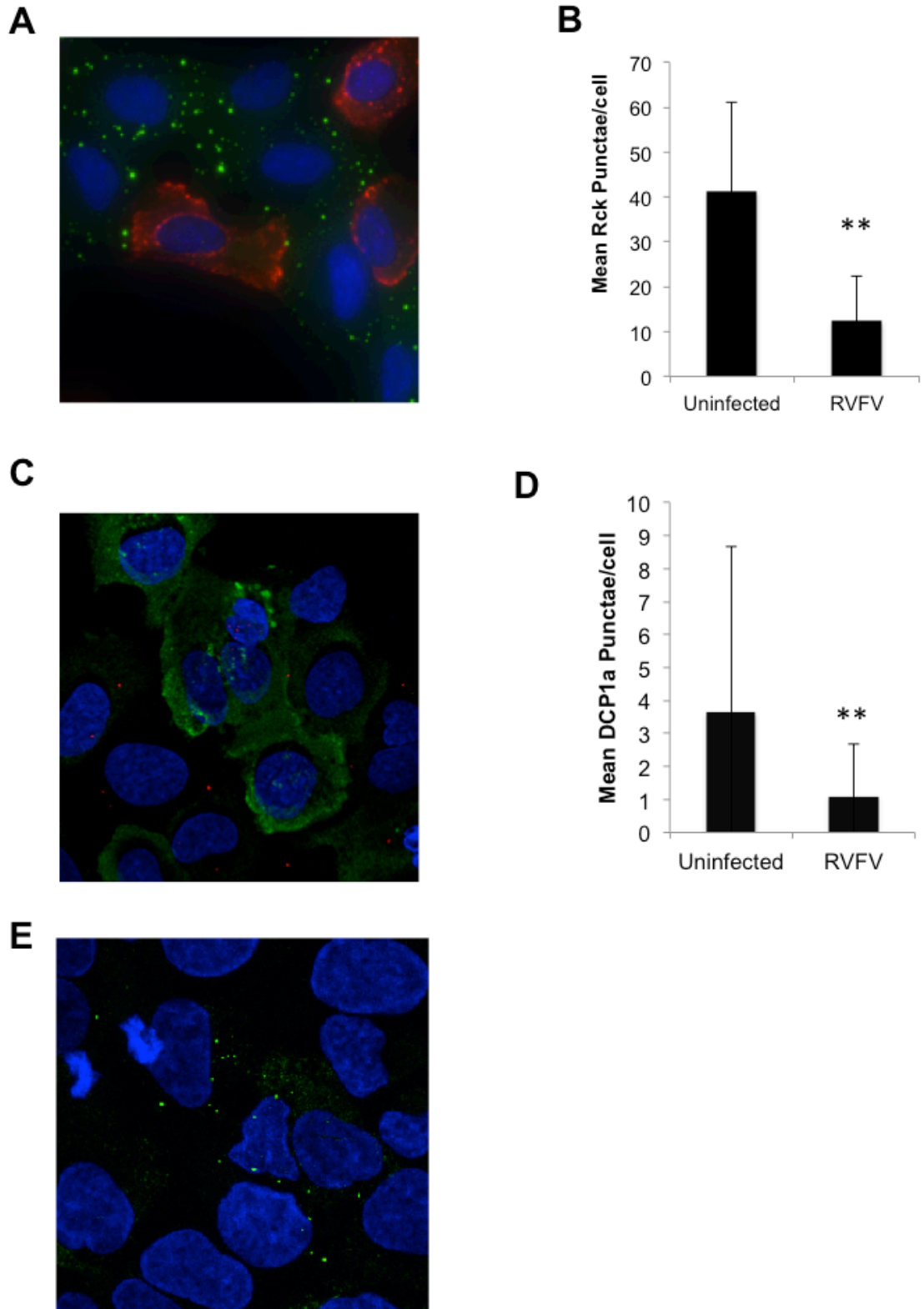
**Figure 4.3. Histone mRNAs are incorporated into RVFV RNAs and limited by decapping.**

**(A)** U2OS cells were treated with the indicated siRNAs and then infected with RVFV (MOI=1). Total RNA was collected 20hpi and analyzed by RT-qPCR for RVFV H2A-N mRNA conjugates. Fold change is normalized to GAPDH (n=3, \*p<0.05). **(B)** U2OS cells were treated with the indicated siRNAs and then infected with RVFV (MOI=1). Total RNA was collected 20hpi and analyzed by RT-qPCR for RVFV HIS3-N mRNA conjugates. Fold change is normalized to GAPDH (n=3). **(C)** Control cells or cells expressing Flag-tagged NUDT16 were infected with RVFV (MOI=1) and total RNA was collected 20 hpi and analyzed by RT-qPCR for RVFV H2A-N mRNA conjugates. Fold change is normalized to GAPDH (n=3, \*p<0.05). **(D)** Control cells or cells expressing Flag-tagged NUDT16 were infected with RVFV (MOI=1) and total RNA was collected 20 hpi and analyzed by RT-qPCR for RVFV HIS3-N mRNA conjugates. Fold change is normalized to GAPDH (n=3, \*\*p<0.01).



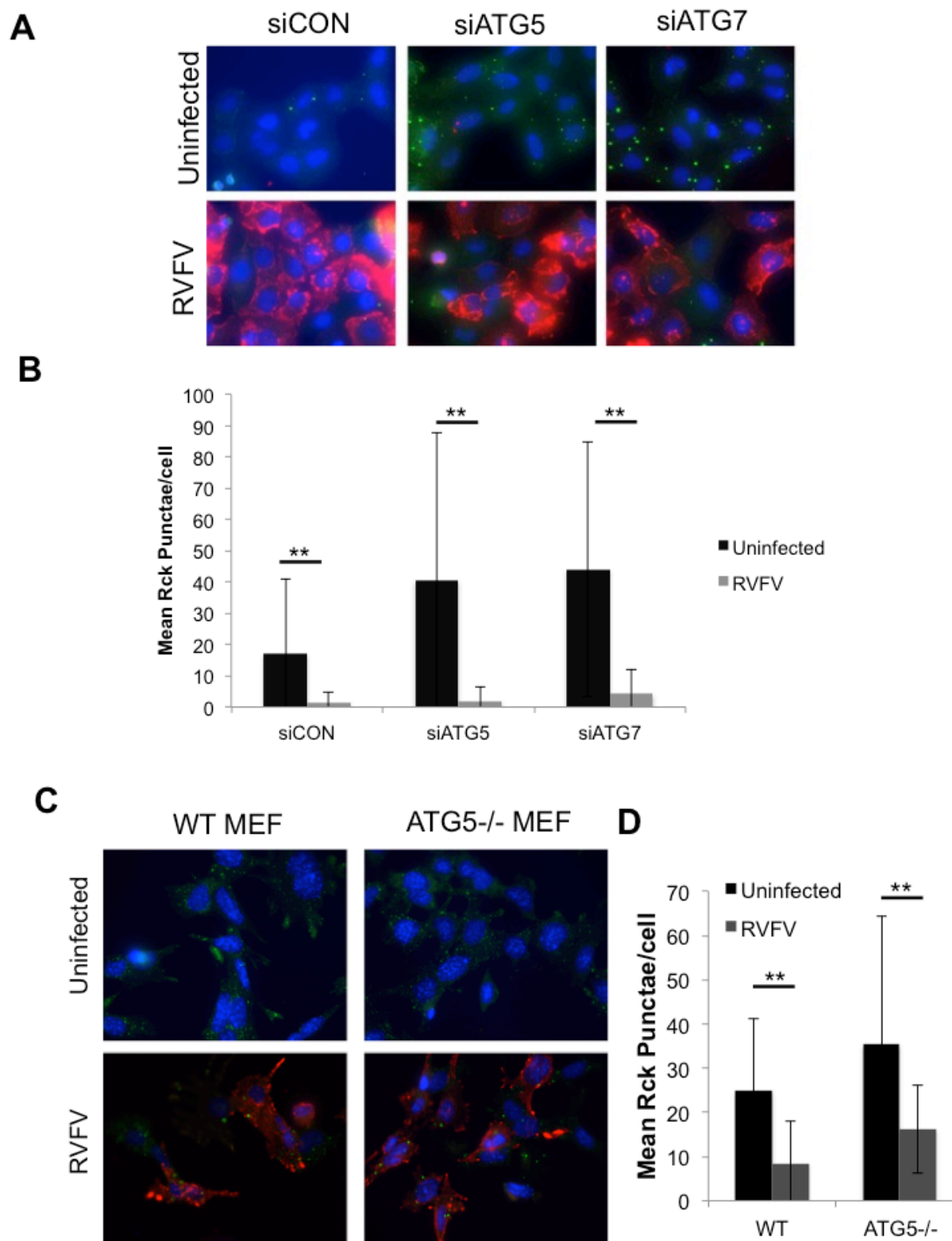
**Figure 4.4. Ribosomal and translationally related mRNAs are targeted by both RVFV cap-snatching and mRNA decapping.** (A) LOGO analysis of the first 10 nt of 83 clones showing

**Figure 4.4 (cont.):** sequence preference of RVFV cap-snatching. Preference for a base is shown as a function of letter size. **(B)** LOGO analysis of the last 10 nt of 83 clones showing sequence preference of RVFV cap-snatching. Preference for a base is shown as a function of letter size. **(C)** LOGO analysis of 83 full-length sequences showing sequence preference of RVFV cap-snatching. Preference for a base is shown as a function of letter size. **(D)** Analysis of enrichment of 49 snatched sequences by cellular GO term. Enrichment of functional categories is shown as a function of P value. **(E)** U2OS cells were treated with the indicated siRNAs and then infected with RVFV (MOI=1). Total RNA was collected 20hpi and analyzed by RT-qPCR for RVFV RPL37A-N mRNA conjugates. Fold change is normalized to GAPDH (n=3, \*p<0.05). **(F)** U2OS cells were treated with the indicated siRNAs and then infected with RVFV (MOI=1). Total RNA was collected 20hpi and analyzed by RT-qPCR for RVFV RPS3A-N mRNA conjugates. Fold change is normalized to GAPDH (n=3, \*p<0.05).



**Figure 4.5. RVFV infection triggers loss of Processing bodies in human cells. (A)** U2OS cells were infected with RVFV (MOI=1) at 14C and virus was allowed to bind for 1 hour; cells

**Figure 4.5 (cont.):** were then washed and incubated at 37C for 11 hours. Cells were fixed and stained for Rck/DDX6 (green), RVFV N protein (red) and total nuclei (blue). **(B)** Quantification of Rck punctae in uninfected versus RVFV infected cells as shown in **A**. (\*\*p<0.01, n=1). **(C)** U2OS cells were treated and infected as in **A**. Cells were fixed and stained for DCP1a (red), RVFV N protein (green) and total nuclei (blue). **(D)** Quantification of DCP1a punctae in uninfected versus RVFV infected cells as shown in **C**. (\*\*p<0.01, n=1). **(E)** Flag-NUDT16 expressing U2OS cells were processed and stained with Alexa-488 conjugated anti-Flag (green) and for total nuclei (blue). A representative image is shown.



**Figure 4.6. RVFV-dependent P body clearance occurs independent of autophagy. (A)** U2OS cells were transfected with the indicated siRNAs and infected three days post transfection with

**Figure 4.6 (cont.):** RVFV (MOI=1) at 14C; virus was allowed to bind for 1 hour. Cells were then washed and incubated at 37C for 11 hours. Cells were fixed and stained for Rck/DDX6 (green), RVFV N protein (red) and total nuclei (blue). **(B)** Quantification of Rck punctae in uninfected versus RVFV infected cells as shown in **A**. (\*\*p<0.01, n=1). **(C)** WT or ATG5<sup>-/-</sup> MEF cells were infected with RVFV (MOI=1) at 14C; virus was allowed to bind for 1 hour. Cells were then washed and incubated at 37C for 11 hours. Cells were fixed and stained for Rck/DDX6 (green), RVFV N protein (red) and total nuclei (blue). **(D)** Quantification of Rck punctae in uninfected versus RVFV infected cells as shown in **C**. (\*\*p<0.01, n=1).



Snatched sequence (5' to 3')	Official Gene Symbol
CTTTCTGGGCTCAC	RPL37A
CTTTTCATTCCC	C17orf76-AS1
TCTGACCAGCAC	RPS3A
CGCCACCGTCGTC	KRT8
CGCGTCGCTAGC	NDUFS5
ACGGCCAGGTTGGGT	AGL
ACTTCCGGTTCCCG	MTRF1L
CTCTTCCGCCGTCAC	EEF2
GTTTTGCAGACGC	PPIA
AAGCGTTGGGTGAC	LOC339240
CTCTGCCACTCTCG	TTC28
CTTTTTCGCA	EEF1A1
TGGCCCCAGCGGTG	IWS1
CTCAGCAGCCAG	DDHD2
TTCTCTCTCGGC	EIF3C
CAGACCGCCGAGG	PCBP1
CTCTCGCCAGGC	RPLP0
GCGGAGCCGCG	HDAC1
CACTCGTGTCTC	BEX1
CTTTTCTCTCTC	BET1
AGTGTGAGGGGC	NOMO1
CTCGGCAGCCGC	SNX29
GGCGCAGAGGCCTGC	HBS1L
CCCCTCTCTGTCTT	DESI1
ACTTGGCTTCAAAGC	CDK1
AGCTGGCCAGGTC	LOC541473
CTCTTTCGCTCAGG	RPS23
GTTTTTCAAAG	DLEU7-AS1
GTTTCGCCTCAGG	DERL1
TTTCTGCCCGTGGACG	HNRNPA1
AGGAGCGTAGAGGC	FAF2
TTCTGGCGCGGAG	MARCKSL1
AGAGTCCGAGCCG	CUL3
CTCTTTCCAGCCAGC	RPS8
CCCTTTCCTCAGC	RPLP1
AGTGCGGGGTCGGC	CTS2
GTTTTGCAGACG	PPIA
CGCCTGGACGCAG	FXYD5
AGTAGCAGCAGCG	TPRXL
CTCTTCCGCCGCC	RABGGTA
GCTGCCGCCGTCGC	CADM2
CTCTTTCCTTC	RPL10
CCCAGACCAGC	PCBP2
ACAAAGCCCAGACG	HNRNPM
AGAGCCGCCATC	ARF1
CTTGAGAGAAGCAAG	GPR61
AGCCGCTGCGCCCGAG	THBS1
TTAGCGACTATTGC	REXO2
ACCGACCAAATGGC	ETAA1

**Table 4.1. Cap-snatched sequences mapped back to human 5'UTRs.**

## V. CONCLUDING REMARKS

### 1. Summary

With climate change increasing the natural range of biting-insect vectors and the encroachment of human development into the habitat of reservoir species, arboviruses are an increasing threat to human health and agriculture worldwide. The lack of antiviral therapeutics and vaccines against these pathogens necessitates that we increase our understanding of their host requirements and immune evasion in order to combat these diseases. Additionally, the unique lifecycle of arboviruses provides an excellent tool to probe deeply conserved biological pathways and how these viruses interact to subvert or utilize these functions. To this end, we have performed a genome-wide RNAi screen in *Drosophila* cells to understand the virus-host interactions of the mosquito-transmitted bunyavirus, Rift Valley Fever virus, and extended these findings to human studies.

Our genome-wide RNAi screen identified 131 host factors that impact the replication of RVFV (strain MP12) in *Drosophila*. We found 124 factors that, when depleted, allowed for increased infection, including multiple members of the 5' to 3' RNA decay pathway: dDCP2, the canonical mRNA decapping enzyme, and two known activators of decapping, Me31B/DDX6 and dLSM7. Additionally, we identified 7 factors whose presence was necessary for efficient viral replication, including Rab5C, which has been previously validated as a required factor for bunyaviral entry (45); taken together with our previous finding that SUPT5H, another antiviral factor present in our gene set, is antiviral against other arboviruses in *Drosophila* (143), the results of this screen have both validated former knowledge of arboviral replication and enriched our understanding of RVFV-host interactions in insects.

As viruses have evolved unique mechanisms of hijacking host factors, unusual molecular structures have evolved to decoy and recruit host proteins. Concomitantly, cells have also evolved ways to detect and destroy these Pathogen Associated Molecular Patterns (PAMPs). One such molecular signature are the unusual RNA species made during viral infection that are not normally found in cells, especially in the case of RNA viruses. A variety of host proteins exist to detect and destroy these unusual RNAs throughout distantly related species. The RNAi silencing machinery in particular has been shown to be potently antiviral in plants and insects, and some viruses have even evolved ways to subvert this pathway. It is possible that other host pathways of RNA decay are utilized in an antiviral fashion, in addition to their basal metabolic functions within the cell. There has been recent increased interest in the understanding of the interaction between viral replication and RNA decay factors. Interactions have been demonstrated between 5' to 3' decay machinery and mosquito-transmitted flaviviruses, such as West Nile virus (WNV) and Dengue virus (DENV). XRN1 and DDX6, which we found to be antiviral factors for RVFV replication, have been shown to be recruited to viral replication sites and required for efficient WNV replication (24), and DDX6 has been shown to bind to DENV viral RNA to facilitate replication (138). These data, along with our finding that VSV and SINV replication are not effected by RNA decapping, suggest that bunyaviral replication is specifically restricted by the RNA decapping machinery.

I demonstrated that RNA decapping in insects restricts bunyaviruses indirectly by creating a bottleneck of mRNA substrates available for cap-snatching. Rather than directly decaying viral RNA species, I found that dDCP2 does not affect viral mRNA cap-status or stability. Rather, dDCP2 restricts infection through the basal metabolism of its own RNA substrates. Immunofluorescence studies revealed that the viral N protein,

essential for cap-snatching, colocalizes with dDCP2 to a high degree in cytoplasmic punctae. Furthermore, profiling of endogenously cap-snatched host messages revealed that these RNAs are stabilized by dDCP2 depletion. Taken together, these results indicate competition between cap-snatching and decapping for a common pool of mRNAs. Additionally, I demonstrated that this restriction is conserved in whole animals (flies) and in mosquito cells in tissue culture and against the distantly related bunyavirus, La Crosse, suggesting that in insects decapping is broadly antiviral and that cap-snatching is an important bottleneck for bunyaviral replication.

Interestingly, sequencing of cap-snatched targets also revealed a preference for cell-cycle-related mRNAs in insects. In line with previous data from humans (144), I showed that P body dynamics are intricately linked to the cell cycle, and that as cells exit S phase and enter G2, P body size and number are significantly increased. This presumably occurs due to the need to degrade mRNAs encoding for DNA replication machinery and cell cycle progression, including replicating histone mRNAs, which are targeted to processing bodies for degradation by decapping. I found that arresting the cell cycle at S/G2, a stage when P bodies are large and enriched for cell cycle mRNAs, dramatically increased RVFV and LACV replication. Indeed, our genome-wide screen identified 28 factors that increased RVFV infection and whose depletion arrests the cell cycle at S/G2. Intriguingly, previous reports have found that in human cells, RVFV causes cell cycle arrest in S phase late during infection and that this cell cycle arrest is advantageous for viral output (6). As the link between P body dynamics and the cell cycle is conserved from insects to humans, I hypothesize that S phase arrest assists RVFV replication through increased targeting of cell cycle mRNAs to P bodies, where they are cap-snatched (Figure 5.1). It has been demonstrated that this cell cycle arrest during viral replication is due to viral activation of the DNA damage response (in spite of

a demonstrated lack of DNA damage during infection) and that chemical inhibitors of this pathway prevent cell cycle arrest and decrease viral replication (6), suggesting that prevention of cell cycle arrest may be a potential therapeutic for RVFV infection.

In mammals, such as mice and humans, at least two distinct cytoplasmic decapping enzymes have been described (117). I demonstrated that both DCP2 and NUDT16, the newly described and more widely expressed decapping enzyme, are antiviral against RVFV in humans. Through both siRNA and overexpression studies, I found that both DCP2 and NUDT16 affect RVFV replication, even with modest levels of depletion or enforced expression. This suggests that RVFV cap-snatches from a common pool of mRNAs targeted by these decappers. Additionally, it suggests that mRNA decapping is under tight regulation, as I see effects on viral replication with low levels of perturbation, and as stable cell lines self selected for cells with low levels of enforced decapper expression.

While both decapping enzymes are capable of restricting RVFV infection, profiling of endogenously cap-snatched targets in human cells revealed interesting effects of individual decappers on endogenous targets. I found that some targets are shared by both DCP2 and NUDT16 and that depletion of either causes increased incorporation of these targets into viral conjugates; interestingly, neither decapper appears able to compensate for the loss of the other, again suggesting that these enzymes are limiting and tightly regulated. Other targets, such as the ribosomal protein mRNA RPS3A, are degraded exclusively by one decapper; while NUDT16 depletion increased the incorporation of RPS3A into viral conjugates, DCP2 knockdown had no effect. This suggests that these enzymes have both redundant and specific mechanisms by which they are activated or through which mRNAs are targeted to them. This may

also suggest both overlapping and specific localization and compartments, which warrants further investigation.

While I did see evidence of cell cycle related mRNAs being endogenously cap-snatched, unlike insect cells, where the primary mRNA targets of viral cap-snatching were cell cycle related, in humans I showed that predominantly, mRNAs related to host mRNA translation were cap-snatched. Intriguingly, during many viral infections in human cells, the host responds by shutting down translation; it has been demonstrated that other bunyaviruses, such as the hantavirus Sin Nombre, encode their own translational initiation activity in the N protein (84). I hypothesize that viral cap-snatching in humans primarily targets translation related mRNAs because they are being targeted for degradation by the host during infection, and that these mRNAs are targeted to areas where both decappers and the cap-snatching machinery compete (Figure 5.2). To this end, I also found that viral infection triggers loss of visible P bodies as measured by multiple markers. It has been demonstrated that some viruses can target stress granule or P body components for degradation or relocalization, thus inhibiting their formation (24, 39), however I found that protein levels of these markers (Dcp1a, Rck/DDX6) and for DCP2 itself are stable during infection, suggesting that some other mechanism causes these morphological changes (data not shown). Activation of decapping could account for these results, as increasing degradation of nucleating mRNAs would lead to the dissociation of microscopically visible punctae. It is also possible that P body mRNAs are limiting enough that viral cap-snatching itself accounts for a decreased ability of P bodies to nucleate. Early work examining the effects of RNA stability during bunyaviral infection demonstrated that RNAs have rapidly decreasing half-lives during LACV infection, and that even more stable mRNAs, such as actin, are degraded at an increased rate (104). It was shown that these effects continue, even if viral transcription

is halted by cycloheximide treatment (104), suggesting that RNA instability is independent of viral cap-snatching and perhaps is the result of increased decapping activity during infection.

Overall, this work advances our understanding of 5' to 3' RNA decay and mRNA decapping from insects to humans. We demonstrate that specific pools are targeted by 5' to 3' RNA degradation, rather than all RNAs, and we hypothesize that these pools are malleable depending on cellular context. Additionally, we find that specific pools of RNAs involved in particular programs (the cell cycle in insects and translation in mammals) are the targets of RVFV cap-snatching during infection and that these RNA pools overlap with decapping targets. This work further demonstrates that intricate interactions exist between RNA virus replication and RNA decay pathways and suggests that RNA decay components may be potential targets for therapeutic intervention in the treatment of RNA viral disease.

## **2. Future Directions**

While we focused on the interplay between mRNA decapping and viral transcription in our genome-wide RNAi screen, which included P body resident proteins and cell cycle genes, we also validated a large number of additional genes that impact RVFV infection. We identified two genes with roles in transcriptional pausing, dSUPT5H and dSUPT6H, which likely regulate the anti-RVFV transcriptional program, as we recently found that pausing controls antiviral defense against diverse viruses, including RVFV (143). Future studies can explore whether the other genes involved in transcription or splicing that we identified regulate antiviral gene expression programs in insects. Furthermore, we identified 6 of the 7 core components of the COPI coatamer as antiviral. COPI is involved in retrograde transport between the golgi and endoplasmic

reticulum, impacting secretion (Figure 3.2, Table 6.1). Although viral budding and egress would be attenuated by blocking secretion, the screen was not dependent on these processes, and therefore, it is unlikely that these steps are involved in the restriction. Furthermore, our readout for infection is expression of a cytoplasmic protein (nucleocapsid), suggesting that retrograde transport likely restricts viral infection prior to translation. Many RNAi screens have identified the COPI coatamer as required for infection, at the level of entry (19, 70) or RNA replication (31, 98), and thus we have potentially uncovered a new aspect of COPI-virus interactions. Further studies will reveal how COPI and the other genes identified impact RVFV replication.

In this gene set, we also identified 40 core components of the ribosome, six genes impacting ribosomal biogenesis and 45 genes with overall impacts on translation as antiviral. While human cells shut down translation in an attempt to combat some viral infections, we found that knockdown of translational factors in insects actually increases infection. Inhibition of translation via polysome disassembly has been shown to increase P body size in *Drosophila* cells (42). I hypothesize that knockdown of translational factors leads to increased mRNA targeting to P bodies, thus increasing P body size and number in a manner consistent with cell cycle arrest. Future studies will confirm whether this panel of genes indeed affects P body morphology; if not, clarification of the interactions between the translational pathway and RVFV in insects should be further dissected, especially since we find particular targeting of ribosomal protein RNAs for viral cap-snatching in human cells.

Our screen set out to identify factors effecting RVFV replication, which led to the discovery that cell cycle mRNAs are targets of both viral cap-snatching and dDCP2-dependent decapping. This suggests pools of cell cycle RNAs are under precise control by dDCP2 in insects; how these particular mRNAs are targeted to dDCP2 remains



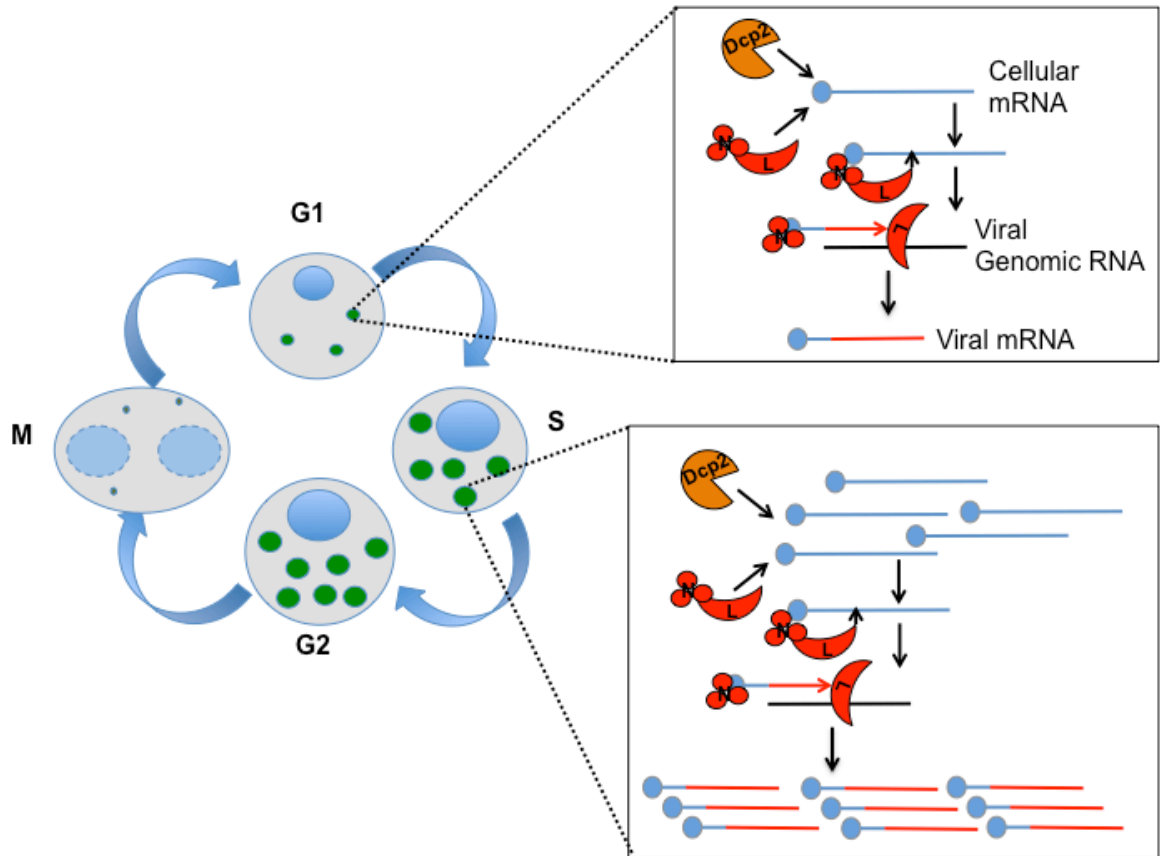
unknown. Furthermore, increasing evidence suggests that P bodies are not compartments of uniform composition, rather they may possess potential specializations since P body components only partially overlap and can be in separate structures (111, 141). Indeed, my own studies found that while dDCP2 tightly co-localizes with RVFV, the canonical binding partner of dDCP2, dDCP1A only partially co-localizes with either dDCP2 or RVFV N (61). It is likely that in addition to specialization in their protein composition, these granules likely vary in their specificity for RNA targets. Therefore RVFV, and other bunyaviruses, may provide a useful tool for probing the RNA composition of subsets of granules.

I profiled mRNA targets of RVFV in rapidly dividing cells. However, many bunyaviruses replicate in senescent cells, such as neurons, and RVFV is neurotropic. Interestingly, neurons possess specialized and extensive P body like structures termed “neuronal granules” that have a similar protein content and function and provide spatial regulation of translation (3). RNAs in these granules are sensitive to overexpression of DCP1A and their expression may affect synaptic plasticity and cytoskeletal organization (97). I hypothesize that neurotropic bunyaviruses use this pool of non-translating stored mRNAs for cap-snatching, and this large pool of targets may explain efficient replication in this cell type. Future studies will examine endogenously cap-snatched mRNA targets in primary rat neurons.

To add further complexity, while *Drosophila* encode only one known decapper, in mammals there are at least two decappers (DCP2 and NUDT16), possessing both specific and redundant functions in mRNA decay pathways (76). This suggests a tight degree of regulation in both the specificity of target selection to decapping enzymes, which likely reside in distinct compartments, and decapping activation itself. I found that both overlap and specificity exist in the mRNAs targeted by these decappers in human

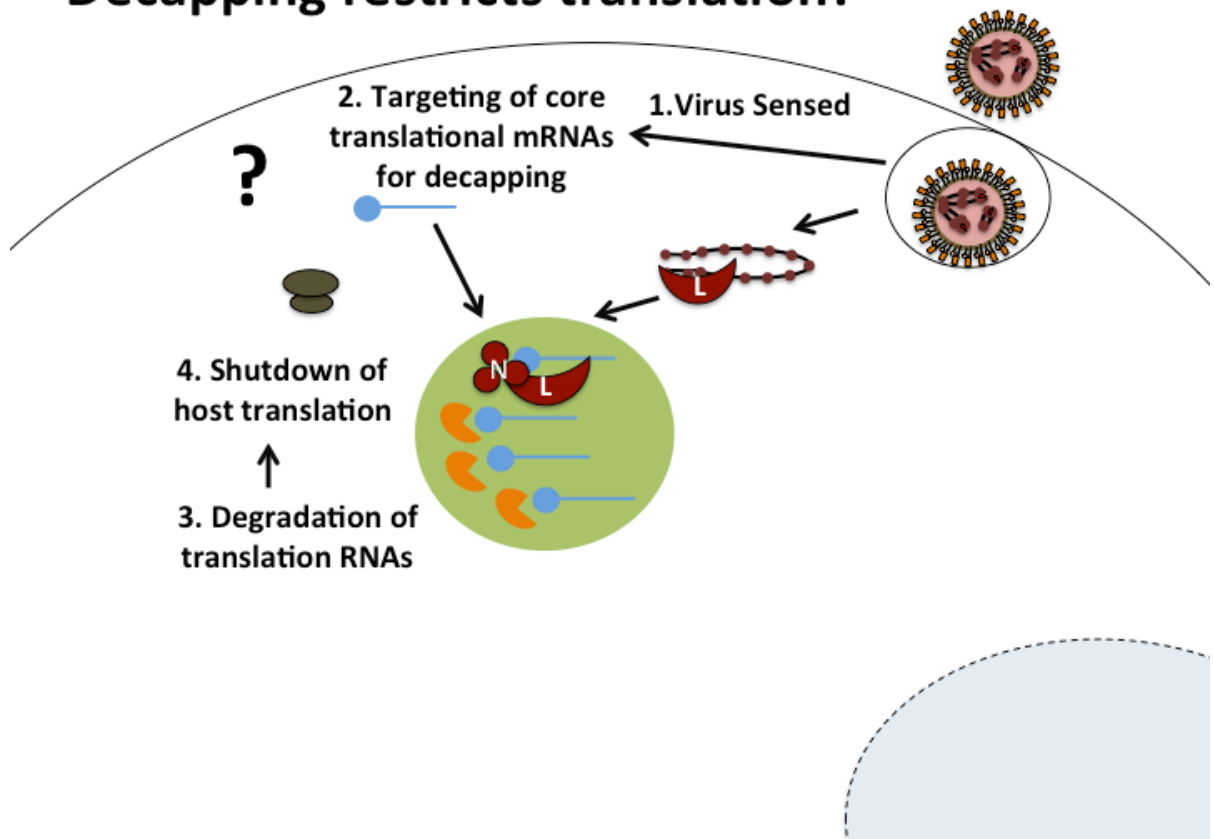
cells and their ability to restrict specific RVFV-host mRNA conjugates. While it has been established that NUDT16 exists both in the nucleus and the cytoplasm, whether it resides in P bodies or P-body-like punctae in the cytoplasm has not been described and is partially hampered by a lack of endogenous antibodies. Preliminary data suggest that, indeed, NUDT16 may exist in cytoplasmic punctae in the cytoplasm, at least in the context of enforced expression (Figure 4.5E). Future work will examine whether these punctae colocalize with known P body markers, such as DDX6 and DCP1a, DCP2 itself, or the viral cap-snatching machinery. I hypothesize that partial overlap exists between all of these compartments and that this spatial organization contributes to the specificity of mRNAs targeted by viral cap-snatching and by decappers. Further work should elucidate the regulation of RNA targeting to decapping enzyme compartments and their activation, as these are potentially druggable proteins. The potential to induce decapping to restrict bunyaviral infection through the decay of RNA targets is exciting, as no therapeutic interventions currently exist for these infections. Additionally, my findings that cell cycle mRNA stability is specifically sensitive to decapping in insects suggests the possibility that decapping could be induced as a mechanism for restricting the expression of cell cycle regulated genes during cell cycle dysregulation, including cancer. While I have shown that ribosomal and translation related mRNAs are primarily targeted for cap-snatching by RVFV in humans, whether the basal levels of these RNAs are appreciably affected by decapping in uninfected cells remains to be established. It is possible that translation mRNAs are targeted specifically for decay through decapping activation as a means to trigger translational shutdown; I hypothesize that this specificity is mediated through targeted decay of TOP motif containing mRNAs. Whether the stability of translation related mRNAs is decay dependent endogenously, or whether this occurs only as a novel response to infection requires further investigation (Figure 5.2). Gene

regulation and control are of the utmost importance in both research and disease, and the technical advances achieved through the discovery of RNAi have revolutionized the field. The discovery that multiple decappers exist with potential target specificity points to new mechanisms for controlling gene programs by harnessing decapping to induce specific cellular mRNA decay.



**Figure 5.1: Decapping and cap-snatching machinery compete for cell cycle regulated mRNAs.** **Left:** Schematic of P body dynamics during cell cycle progression. During G1, P bodies (green) are present at low levels in cells. As cells exit S phase and progress into G2, P bodies increase in number and size (61, 144). As cell enter mitosis, P bodies are lost (144). **Upper Inset:** RVPV cap-snatching and Dcp2 mRNA decapping are competing processes. Viral mRNA transcription initiates upon the binding of RVPV N (red circles) to 5'caps of cellular mRNAs (blue). Next, RVPV L (red moons) is recruited and its endonuclease activity cleaves 10-18bp downstream of the cap and uses this primer to initiate viral transcription from the genomic RNA (black line), producing cellular-virus conjugate mRNAs (blue and red line). Dcp2 (orange) targets and degrades the same pool of cellular mRNAs that RVPV uses for transcription, creating a bottleneck. **Lower Inset:** During the S/G2 phase of the cell cycle, as mRNAs required for DNA replication are targeted for degradation (blue), this increased level of substrates alleviates the bottleneck, allowing the viral cap-snatching machinery to increase viral transcription (blue and red lines). Thus, arresting cells in S/G2 increases bunyaviral replication.

## Decapping restricts translation?



**Figure 5.2: Decapping may restrict viral infection in humans through the decay of core translation mRNAs.** 1. During RVFV infection, the viral infection is sensed. 2. A signaling cascade may potentially lead to the targeting of core translational mRNAs for decapping. 3. Following targeting, core translation mRNAs are degraded. 4. Decay of host translation mRNAs over time leads to loss of ribosome biogenesis and shutdown of host translation.

**Table 6.1. Validated hits from a genome-wide RNAi screen for genes impacting RVFV replication in *Drosophila*.** Percent infection Z scores of screen hits with human homolog names, Flybase IDs, and informatics based cellular categories. Genes with cell cycle GO terms, S phase associated GO terms, or those that showed >30% increase in nuclear size (large nuclei) are indicated (n=2, p<0.05).

Flybase Symbol	Human Symbol	PRIMARY SCREEN % INFECTION		SECONDARY SCREEN % INFECTION		Flybase Gene ID	Placement in Figure 3.2	Cell Cycle GO term	S phase associated	Large nuclei
		Z SCORE 1	Z SCORE 2	Z SCORE 1	Z SCORE 2					
alphaCop	COPA	1.76	3.20	4.13	3.58	FBgn0025725	COPI			
ATPsyn-beta	ATP5B	1.37	2.37	6.30	5.55	FBgn0010217	Mitochondrial			
ATPsyn-gamma	ATP5C1	2.93	2.68	5.69	6.33	FBgn0020235	Mitochondrial			X
Bap60	SMARCD1	1.76	3.46	3.41	3.33	FBgn0025463	Transcription/Chromatin			
beta'Cop	COPB2	2.24	2.93	2.76	3.77	FBgn0025724	COPI			
betaCop	COPB1	1.83	2.69	7.00	6.72	FBgn0008635	COPI			X
Bx42	SNW1	3.44	2.04	4.05	4.76	FBgn0004856	Transcription/Chromatin			
c12.1	CWC15	3.85	1.45	3.49	4.50	FBgn0040235	Splicing			
cdc2	CDK1	2.64	2.93	5.51	6.35	FBgn0004106	Cell Cycle	X		X
CG10754	SF3A2	2.16	1.72	5.74	5.52	FBgn0036314	Splicing	X		X
CG11583	BRIX1	1.59	4.88	3.79	4.92	FBgn0035524	Ribosome Biogenesis			
CG13054		2.39	1.53	1.50	1.51	FBgn0036584	Other			
CG13096	RSL1D1	1.58	3.06	Ribosome		FBgn0032050	Ribosome			
CG1311	SLC44A1	1.59	4.88	3.86	3.20	FBgn0035523	Membrane			
CG13705		1.90	1.38	1.70	1.99	FBgn0035582	Other			
CG14434		1.96	1.64	1.40	2.45	FBgn0029915	Other			
CG14641	RBM22	1.89	1.64	5.55	5.50	FBgn0037220	Splicing			
CG14667		1.42	3.74	1.55	1.31	FBgn0037317	Nucleic Acid Binding			
CG15220	RPA3	1.68	2.14	3.74	4.37	FBgn0030322	Cell Cycle		X	
CG1542	EBNA1BP2	1.74	4.10	4.24	5.27	FBgn0039828	Cell Cycle			
CG16903	CCNL1	2.50	3.82	6.27	4.56	FBgn0040394	Cell Cycle			X
CG16941	SF3A1	2.88	2.37	4.99	4.48	FBgn0038464	Splicing	X		

CG1746	ATP5G2	2.36	3.04	3.21	3.46	FBgn0039830	Mitochondrial			
CG18577		1.31	1.32	1.93	2.25	FBgn0037870	Other			
CG2063	SAP30BP	1.58	4.43	5.48	5.40	FBgn0033400	Splicing			X
CG3224	ZNF593	4.69	1.92	3.39	3.39	FBgn0029885	Nucleic Acid Binding			
CG32808	KLK7	1.61	4.29	2.75	2.88	FBgn0052808	Protein Modifications			
CG40127	RNASEK	-2.01	-1.61	-5.14	-7.43	FBgn0262116	RNA decay			
CG4849	EFTUD2	4.78	2.87	6.78	5.86	FBgn0039566	Splicing			
CG6841	PRPF6	1.93	3.82	4.43	4.84	FBgn0036828	Splicing			
CG7058		1.37	2.23	1.76	1.46	FBgn0030961	Other			
CG7675	RDH14	1.66	1.55	3.50	1.37	FBgn0038610	Metabolism			
CG8097	DALRD3	1.32	1.50	1.77	1.71	FBgn0030660	Translation			
CG8108	CIZ1	2.26	1.96	2.03	1.83	FBgn0027567	Cell Cycle		X	
CG8636	EIF3G	2.60	3.89	5.40	5.39	FBgn0029629	Translation	X		
CG8801	GTPBP4	1.93	3.29	3.38	4.32	FBgn0028473	Ribosome Biogenesis			
CG9667	ISY1	2.87	3.18	5.71	4.98	FBgn0037550	Splicing			
CG9715		2.29	3.04	3.53	3.38	FBgn0036668	Nucleic Acid Binding			X
Chro		1.45	2.87	2.01	2.69	FBgn0044324	Cell Cycle			
CycA	CCNA1	2.29	3.56	9.35	9.50	FBgn0000404	Cell Cycle	X	X	X
Dcp2	DCP2	2.12	3.52	4.57	5.08	FBgn0036534	RNA decay			
deltaCOP	ARCN1	1.95	2.89	COPI Transport		FBgn0028969	COPI			
Dis3	DIS3	1.52	3.35	5.04	4.56	FBgn0039183	RNA decay			X
DMAP1	DMAP1	1.49	2.34	3.47	4.01	FBgn0034537	Transcription/Chromatin	X		
E2f	E2F3	1.97	2.51	2.43	2.16	FBgn0011766	Transcription/Chromatin	X		
eIF-3p66	EIF3D	1.69	3.79	6.02	7.05	FBgn0040227	Translation			
eIF2B-beta	EIF2B2	1.33	1.73	4.51	5.80	FBgn0024996	Translation			
gammaCop	COPG	2.15	3.03	COPI Transport		FBgn0028968	COPI			
geminin	GMNN	2.50	4.96	8.27	7.98	FBgn0033081	Cell Cycle		X	X

His3:CG31613	HIST2H3C	2.60	1.85	2.78	3.45	FBgn0051613	Cell Cycle		X	
His4:CG31611	HIST1H4B	1.52	1.95	3.55	3.97	FBgn0051611	Cell Cycle		X	
hoip	NHP2L1	2.27	3.63	5.77	6.26	FBgn0015393	Splicing	X		X
l(1)1Bi	MYBBP1A	2.26	1.81	2.50	2.67	FBgn0001341	Ribosome Biogenesis			
l(2)37Cc	PHB	1.60	2.66	4.41	5.20	FBgn0002031	Mitochondrial			
LSm7	LSM7	1.36	1.96	5.27	5.12	FBgn0261068	RNA decay			
me31B	DDX6	2.01	3.54	5.74	6.41	FBgn0004419	RNA decay			
mRpL27	MRPL27	2.29	3.84	Ribosome		FBgn0053002	Mitochondrial			
mts	PPP2CB	2.36	1.68	5.08	4.54	FBgn0004177	Cell Cycle	X		
nej	EP300	1.89	4.13	4.68	4.37	FBgn0261617	Cell Cycle			
Nmt	NMT1	1.43	1.40	3.92	4.94	FBgn0020392	Protein Modifications			
ns1	GNL3L	2.27	5.19	4.13	5.51	FBgn0038473	Ribosome Biogenesis			
Nup62	NUP62	2.14	3.58	5.95	5.50	FBgn0034118	Nuclear Pore			X
Pp4-19C	PPP4C	2.22	2.69	3.72	3.27	FBgn0023177	Cell Cycle	X		
Pros25	PSMA2	-2.14	-2.22	-6.46	-7.40	FBgn0086134	Proteasome	X		
Pros26	PSMB1	-3.92	-3.20	-7.51	-5.11	FBgn0034118	Proteasome			
Prosbeta3	PSMB3	-2.81	-2.13	-4.41	-6.34	FBgn0026380	Proteasome			X
Prosbeta5	PSMB5	-1.91	-2.01	Proteasome		FBgn0029134	Proteasome	X		
Prp18	PRPF18	1.53	2.87	4.44	4.89	FBgn0027784	Splicing			
qm	RPL10	2.21	3.64	Ribosome		FBgn0019662	Ribosome			
Rab5	RAB5A	-2.96	-3.18	-3.84	-3.50	FBgn0014010	Trafficking	X		X
Rbm13	MAK16	2.11	4.15	3.23	3.34	FBgn0030067	Ribosome Biogenesis	X		
Rca1		1.83	4.73	6.56	4.91	FBgn0017551	Cell Cycle	X	X	X
RnrS	RRM2	1.61	1.30	5.13	5.49	FBgn0011704	Cell Cycle		X	X
RpA-70	RPA1	1.70	2.78	5.56	5.71	FBgn0010173	Cell Cycle	X	X	X
RPA2	RPA2	1.74	5.43	3.03	3.27	FBgn0032906	Cell Cycle		X	X
Rpb11	POLR2J	3.47	1.69	5.08	3.42	FBgn0032634	Transcription/Chromatin			



Rpb5	POLR2E	1.39	3.78	4.65	5.16	FBgn0033571	Transcription/Chromatin			X
Rpb7	POLR2G	1.61	3.51	4.09	5.94	FBgn0051155	Transcription/Chromatin			X
Rpb8	POLR2H	2.98	2.04	6.06	5.54	FBgn0037121	Transcription/Chromatin			X
Rpl140	POLR2B	1.38	3.19	5.78	5.77	FBgn0262955	Transcription/Chromatin			X
Rpl215	POLR2A	2.98	3.48	5.65	6.57	FBgn0003277	Transcription/Chromatin			
RpL10Ab	RPL10A	4.59	4.40	Ribosome		FBgn0036213	Ribosome	X		
RpL11	RPL11	1.68	4.44	Ribosome		FBgn0013325	Ribosome	X		
RpL12	RPL12	2.80	3.32	Ribosome		FBgn0034968	Ribosome	X		
RpL13	RPL13	1.49	3.92	Ribosome		FBgn0011272	Ribosome	X		
RpL15	RPL15	1.58	2.82	Ribosome		FBgn0028697	Ribosome			
RpL17	RPL17	1.93	2.80	Ribosome		FBgn0029897	Ribosome	X		
RpL18A	RPL18A	1.93	4.07	Ribosome		FBgn0010409	Ribosome	X		
RpL19	RPL19	2.63	4.41	Ribosome		FBgn0002607	Ribosome	X		
RpL21	RPL21	1.35	3.84	Ribosome		FBgn0032987	Ribosome	X		
RpL22	RPL22	1.90	2.90	Ribosome		FBgn0015288	Ribosome	X		
RpL23	RPL23	2.46	4.40	Ribosome		FBgn0010078	Ribosome	X		
RpL23A	RPL23A	3.22	4.86	Ribosome		FBgn0026372	Ribosome			
RpL24	RPL24	2.63	3.38	Ribosome		FBgn0032518	Ribosome	X		
RpL26	RPL26	4.73	3.00	Ribosome		FBgn0036825	Ribosome	X		
RpL27A	RPL27A	2.29	3.84	Ribosome		FBgn0261606	Ribosome			
RpL28	RPL28	1.57	1.89	3.16	2.42	FBgn0035422	Ribosome	X		
RpL3	RPL3	1.88	3.68	Ribosome		FBgn0020910	Ribosome	X		
RpL30	RPL30	2.45	3.80	Ribosome		FBgn0086710	Ribosome	X		
RpL31	RPL31	2.39	4.56	Ribosome		FBgn0025286	Ribosome	X		
RpL32	RPL32	4.13	2.73	Ribosome		FBgn0002626	Ribosome	X		
RpL34a	RPL34	2.76	3.98	Ribosome		FBgn0039406	Ribosome			
RpL34b	RPL34	2.37	4.51	Ribosome		FBgn0037686	Ribosome			

RpL35A	RPL35A	3.70	1.73	Ribosome		FBgn0037328	Ribosome			
RpL36	RPL36	2.21	4.14	Ribosome		FBgn0002579	Ribosome			
RpL36A	RPL36AL	2.47	4.95	Ribosome		FBgn0031980	Ribosome	X		
RpL37a	RPL37	2.77	5.68	Ribosome		FBgn0030616	Ribosome			
RpL37A	RPL37A	2.81	5.00	Ribosome		FBgn0261608	Ribosome			
RpL38	RPL38	1.62	4.61	Ribosome		FBgn0040007	Ribosome			
RpL39	RPL39L	2.38	2.65	Ribosome		FBgn0023170	Ribosome	X		
RpL4	RPL4	2.80	4.70	Ribosome		FBgn0003279	Ribosome			
RpL40	UBA52	1.74	3.65	Ribosome		FBgn0003941	Ribosome			
RpL5	RPL5	1.92	3.65	Ribosome		FBgn0064225	Ribosome			
RpL6	RPL6	2.36	4.72	Ribosome		FBgn0039857	Ribosome			
RpL7	RPL7	1.89	4.08	Ribosome		FBgn0005593	Ribosome	X		
RpL7-like	RPL7	2.05	2.83	Ribosome		FBgn0032404	Ribosome	X		
RpL9	RPL9	2.12	2.49	Ribosome		FBgn0015756	Ribosome	X		
RpLP0	RPLP0	2.36	2.84	Ribosome		FBgn0000100	Ribosome			
RpLP2	RPLP2	5.20	3.23	Ribosome		FBgn0003274	Ribosome			
RpS21	RPS21	1.72	2.87	Ribosome		FBgn0015521	Ribosome			
RpS4	RPS4X	1.78	4.28	Ribosome		FBgn0011284	Ribosome	X		
Sam-S	MAT2A	2.43	4.33	1.98	2.71	FBgn0005278	Metabolism			X
SmB	SNRPB	2.04	1.46	5.86	5.78	FBgn0262601	Splicing			X
smt3	SUMO3	6.01	2.93	4.90	5.60	FBgn0264922	Protein Modifications			X
Spc105R	CASC5	2.02	1.57	5.59	5.03	FBgn0037025	Cell Cycle	X		X
Spt5	SUPT5H	1.67	3.28	4.49	4.34	FBgn0040273	Transcription/Chromatin	X		
Spt6	SUPT6H	2.46	2.83	4.58	5.96	FBgn0028982	Transcription/Chromatin			X
Spx	SF3B4	1.32	2.73	5.19	4.69	FBgn0015818	Splicing	X		
Syx7	STX7	-2.47	-4.72	-8.90	-6.59	FBgn0086377	Trafficking			
Tango4	PLRG1	3.16	3.19	6.16	5.48	FBgn0030365	Cell Cycle			X

zetaCOP	COPZ1	2.57	3.15	COPI Transport	FBgn0040512	COPI			
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